Sex Differences in the Role of GPER1 in the Dorsolateral Striatum on Motivation and Reward

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Psychology)
in the University of Michigan
2021

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Dedication

This dissertation is dedicated to my mother, Angela Quigley.

Acknowledgements

I would like to thank my esteemed advisor, Dr. Jill Becker for her invaluable mentorship, guidance, and support during the course of my PhD. I would also like to thank members of my dissertation committee, Dr. Kent Berridge, Dr. Natalie Tronson, and Dr. Carol Elias, for their intellectual contributions and sustained commitment to my success and development as a scientist.

I would like to thank the team of research scientists, made up of dedicated staff and eager students, whom I have has the pleasure of working with on these projects. Thank you to the Becker Laboratory technicians, Brandon Luma, Hannah Epstein, and Molly Logsdon for their daily support in all things administrative. I'd also like to acknowledge the tremendous amount of work contributed by undergraduate students whom I mentored in the over the past five years: Laurel Aberle, Benjamin Lipkin, Lahin Lalani, Abigail Camiener, Megan Mulhinch, Jacob Rainey, Aakash Srikanth, Brianna Graham, Kendra Beudoin, Jessica Erkkila, and Nicholas Brdar. Watching you, as my students, grow into independent scientists and succeed in your own academic paths has brought me a tremendous amount of happiness and fulfillment.

I would like to acknowledge Christopher Turner and Ivette Gonzalez, for being my peers and co-graduate students in the Becker Lab. Additionally, I would like to thank former Becker Lab graduate student, Katie Yoest. Katie began as a lab mate but quickly turned into my mentor, my best friend, and someone I consider to be my sister. I am

profoundly thankful for the guidance Katie has given me, as it has been a significant contribution to my success.

I would like to thank the entire biopsychology area, faculty and students, for being my academic family and home for the past five years. I am eternally grateful for the professional relationships and friendships that I had made with you. In particular, Hannah Baumgartner, Cassandra Avila, Sofia Carerra, Sarah Westrick, Mena Davidson, Carlos Vivaldo, Patsy Delacey, Crystal Carr, Sofia Lopez, and Lauren Rysztak for being integral parts of my support system. Lastly, I would like to thank Caitlin Posillico, for my being the person that I can always count on and go to for help. I admire her generosity and selflessness, and I feel lucky to call her my best friend.

I cannot begin to express my appreciation for my fiancé, Brook Boelter, who put his life and career on hold to help me chase my dream of being a PhD. The kindness, patience, and loyalty you have given me is unimaginable and I cannot wait to marry you and give you the same.

Above all, I owe a debt of gratitude to my mother, Angela Quigley. I can never repay you for the many sacrifices that you made to ensure that I could attend college and pursue a PhD. You gave me my motivation, my work ethic, and my understanding of the power of education. Everything that I do is to make you proud. I love you.

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Abstract

There are sex differences in susceptibility to psychostimulant addiction, supported by clinical evidence and pre-clinical research models. In rodent models, female rats acquire a preference for cocaine at lower doses, escalate self-administration behaviors more rapidly, and are more motivated to attain cocaine than males. These heightened addiction-like behaviors in females are modulated by the presence of the gonadal hormone estradiol. The role of estradiol in regulating male drug-seeking behaviors remains unexplored.

In females, estradiol enhances the rewarding and motivating properties of psychostimulants by potentiating drug-induced dopamine release in the dorsolateral striatum (DLS), a brain region implicated in mediating habitual drug-seeking. While ERα, ERβ, and GPER1 are all localized in the dorsal striatum, the majority of research investigating how estradiol alters behavior, in either sex, has focused on ERα and ERβ and not GPER1. The goal of this dissertation is to fill this gap by investigating the role of GPER in the DLS in mediating addiction-like behaviors in both males and females.

In chapter II, the effects of activation or blockade of GPER1 on preference for rewarding stimuli are investigated. In males, GPER1 bi-directionally modulates cocaine preference: activation of GPER1 attenuates cocaine conditioned place preference and inhibition of GPER1 enhances it. GPER1 activation also attenuates males' preference for saccharin solution, suggesting that the effects of GPER1 on reward modulation are not constrained to drugs of abuse. Interestingly, there are no behavioral consequences

of GPER1 activation on cocaine or saccharin preference for females. Levels of ERα, ERβ, and GPER1 in the dorsal striatum are also explored in this chapter. While there are no sex differences in estradiol receptor expression, relative mRNA levels of GPER1 are greater than both ERα and ERβ, for both sexes.

In chapter III, the impact of DLS GPER1 activation on animals' motivation for cocaine and drug-induced reinstatement are assessed. Activation of GPER1 potentiates females' motivation for cocaine. GPER1 activation also causes females to show greater drug induced reinstatement. There is no effect of GPER1 activation on males' propensity to self-administer cocaine, via measurement of motivation, extinction, or drug induced reinstatement.

In chapter IV, the impact of pharmacological activation of DLS-GPER1 on neuronal activation, with and without cocaine exposure, is measured. c-Fos immunoreactivity is used as a proxy for neuronal activation throughout different brain regions. Intra-DLS GPER1 activation attenuates cocaine induced c-Fos in the dorsomedial striatum. In subregions of the ventral striatum, DLS-GPER1 activation or cocaine alone causes increases in c-Fos immunoreactivity levels, but together, do not have compound effects on neuronal activation.

These are among the first studies to identify a novel role for estradiol receptors mediating reward in males. While estradiol increases females' susceptibility towards addiction, activation of GPER1 may be protective against the rewarding effects of drugs of abuse for males. Together, these studies are yet another example of why sex should be considered as a biological variable in experimental research, as it will lead to the sex specific therapeutic targets for disorders, such as addiction.

Chapter I
Introduction

General Overview

Sexual differentiation and organization of brain circuits are mediated by the presence of gonadal hormones during development. During adulthood, these hormones have activational effects on the sexually differentiated brain and mediate sexually dimorphic behaviors. Briefly, during fetal development, testosterone produced by the testes is converted to estradiol by the aromatase enzyme and thus, estradiol is the primary hormone which mediates masculinization of the brain (McCarthy, 2008; Schulster, Bernie, & Ramasamy, 2016). (McCarthy, 2008). In females, the absence of these gonadal steroids actively represses DNA methylation and masculinization, allowing for feminization of the brain to occur (Nugent et al., 2015). Through the actions of estradiol signaling, altered gene expression causes differential patterns of neuronal cell death, growth, and connectivity that have lasting effects on neural circuitry and behavior (Forger, 2006). Namely, estradiol-dependent sexual differentiation of striatal regions, which are implicated in regulating motivation and reward-processing (Cao, Willett, Dorris, & Meitzen, 2018; Meitzen, Meisel, & Mermelstein, 2018).

In the adult brain, estradiol acts within the dopaminergic reward circuitry to alter motivation and goal directed behaviors that are important for reproductive success in females. While this system is adapted to enhance an individual's propensity to perform evolutionarily advantageous behaviors, it is also altered by repeated exposure of drugs of abuse. Previous work has established that in the presence of estradiol increases

female's vulnerability toward addiction by enhancing drug-induced dopamine levels in the brain and motivation for drugs of abuse (Becker & Rudick, 1999; Becker, 2009; Yoest, Cummings, & Becker, 2019a). The role of estradiol in male's drug abuse liability remains unclear.

As an introduction to this dissertation, I will begin by presenting the clinical evidence for female's enhanced vulnerability for addiction and briefly outline the evidence for sex differences within the major theories of addiction. I will then explain the role of estradiol in enhancing drug-induced dopamine neurotransmission and the accompanying changes in drug-seeking behaviors seen in females. Next, I will provide a comprehensive review of estradiol receptor signaling as well as the localization of estradiol receptors throughout the male and female brain. Lastly, I will introduce how the chapters of this dissertation relate to the overall goal of understanding how estradiol, via activation of estradiol receptors, affects males' and females' propensity towards addiction.

Sex differences in addiction

Clinical evidence of sex differences in addiction

Addiction has been categorized into different "stages" and there are sex differences in each stage (Becker & Koob, 2016). Initiation of drug use for women is often driven by psychological factors such as anxiety and depression, or after experiencing negative life events; whereas more men report initial drug use in social settings (Annis & Graham, 1995; Brady & Randall, 1999). Continued drug use causes neuroplastic changes in the reward system and in stress mechanisms in the brain of males and females, which contribute to sex differences in drug-seeking after initial use.

Women who have sought treatment for addiction report their drug consumption escalated more rapidly than do men in treatment, this phenomenon of rapid escalation of drug use in women is known as “telescoping” (Becker & Chartoff, 2019; Haas & Peters, 2000). After escalation of drug use, during maintenance, an individual is constantly thinking about obtaining the next drug dose (Becker & Chartoff, 2019). With continued use of a drug there is a transition to chronic substance use disorder which is characterized by repeated attempts at abstinence and relapse (American Psychiatric Association, 2013; Koob & Le Moal, 2006). During abstinence, women report greater craving than do men, which is modulated to some extent by their hormone cycles (Weinberger et al., 2015). Finally, women are more sensitive to environmental cues and report more spontaneous relapse (Janes et al., 2010). One aspect of the environment that is key to sex differences in addiction is activation of the stress axis. After we have discussed sex differences in the neural systems mediating addiction, we will return to how the stress system interacts with these systems differentially in males and females to put everything in context.

Sex differences within different theories of addiction

Dopaminergic neurons within the ascending mesotelencephalic pathway are activated in response to adaptive rewarding stimuli, such as food consumption, sexual behavior, and social interactions, all of which are necessary functions for health and reproductive success (Everitt et al., 1999; Robinson, Fischer, Ahuja, Lesser, & Maniates, 2016; W. Schultz, 1986; Wise & Rompre, 1989). Drugs of abuse also induce dopamine neurotransmission and sustained drug use causes numerous temporary and permanent physiological changes in the brain (Engel & Jerlhag, 2014; Koob & Le Moal,

2008; Kuczenski, Segal, & Todd, 1997; Robinson & Becker, 1986; Seiden, Sabol, & Ricaurte, 1993; Strakowski, Sax, Setters, & Keck, 1996). The various theories of how dopamine regulates motivated behaviors were developed in male animals however, there are implications for sex differences in vulnerability and propensity towards addiction within the context of these different theories.

The incentive sensitization theory posits that repeated psychostimulant exposure results in sensitization of dopamine neurons which increases 'wanting' of the drug. (Robinson & Berridge, 1993). These neuroadaptations also increase the salience of drug cues which underlie the drive from casual drug use to compulsive drug taking (Berridge, 2007; Robinson & Becker, 1986; Robinson & Berridge, 1993). Females are more susceptible to incentive sensitization than are males, which may explain the enhanced vulnerability of females' transition from intermittent drug use to chronic use (Kawa & Robinson, 2019). Repeated exposure to psychostimulants also causes behavioral sensitization (Robinson & Becker, 1986; Strakowski et al., 1996). Though both males and females show behavioral sensitization to psychostimulants, females exhibit greater enhancement in rotational movements and stereotyped behaviors (i.e. behavioral sensitization), than males do, after repeated amphetamine or cocaine administration (Camp & Robinson, 1988; Robinson, 1984; van Haaren & Meyer, 1991). Females also sensitize at lower doses of cocaine than males (Post, Lockfeld, Squillace, & Contel, 1981). Sensitization is regulated by circulating estradiol in females (Becker, Molenda, & Hummer, 2001; Hu & Becker, 2003; Souza et al., 2014). Intact female rodents show varying degrees of behavioral sensitization based on levels of gonadal hormones during their estrous cycle (Becker & Cha, 1989; Becker, Robinson, & Lorenz,

1982; Morissette & Di Paolo, 1993; Sell, Scalzitti, Thomas, & Cunningham, 2000). This effect of estradiol to enhance sensitization is not seen in males (Becker et al., 2001). Furthermore, testicular hormones do not regulate sensitization in males (Camp, Becker, & Robinson, 1986; Hu & Becker, 2003). Thus, sex differences in sensitization of the ascending dopamine system is a candidate to mediate sex differences in the neural mechanisms of addiction.

An alternate theory is the opponent process theory of addiction which proposes that addiction emerges due to avoidance of withdrawal and the related anhedonia (Koob & Le Moal, 2008; Solomon & Corbit, 1974). In this theory, an initial pleasurable "high" accompanies drug use, which drives motivation for reuse. Over time sustained drug use results in tolerance to the pleasurable effects of the drug and a transition to increased unpleasant effects of withdrawal. Eventually, motivation for continued use is sustained to avoid the unpleasant effects of drug withdrawal (Koob, Caine, Parsons, Markou, & Weiss, 1997). Women report enhanced negative aspects of withdrawal effects from psychostimulants, along with most other classes of drugs (Becker, 2016; Becker, Perry, & Westenbroek, 2012; Brady & Randall, 1999). The severity of withdrawal is reported to be cyclic with gonadal hormones, suggesting that estradiol is mediating both the positive and negative effects of drug use for women (Ruda-Kucerova et al., 2015). Unexplained by this theory, however, is the fact that relapse occurs long after drug withdrawal symptoms subside (Kerstetter, Aguilar, Parrish, & Kippin, 2008; Ruda-Kucerova et al., 2015). Spontaneous relapse also occurs disproportionally in females compared to males (Ruda-Kucerova et al., 2015). Thus, while sex differences in withdrawal likely contribute to sex differences in the pattern of drug taking behavior and

relapse, the opponent process theory alone is not sufficient to explain all of the sex differences reported in substance use disorders.

Finally, risky decision making is associated with enhanced dopamine release dynamics in the nucleus accumbens shell (Freels, Gabriel, Lester, & Simon, 2020). Decision-making and risk-taking are related to the choice to consume drugs of abuse. Males are more likely to make “risky” choices in order to receive a higher value reward (Orsini, Willis, Gilbert, Bizon, & Setlow, 2016). Various studies have investigated the role of the ovarian cycle on decision-making in females and reported no effect (Georgiou et al., 2018; Orsini et al., 2016). The stability of females decision making, including their inability to enhance performance on risk-related tasks across training session compared to males, may be due to their hypersensitivity to punishment (Mohebi et al., 2019). On the other hand, ovariectomy increased risky decision making in females, and estradiol reversed this effect, demonstrating that ovarian hormones maintain this sex difference (Orsini et al., 2020). In women, the sex difference of reduced risk taking may be reflected in the pattern of drug use, where women are more likely to take drugs of abuse or relapse due to stress and lack of social support, compared to men (Becker, McClellan, & Reed, 2017).

Behavioral paradigms to assess reward and motivation

Cocaine enhances monoamine signaling by blocking dopamine, serotonin, and norepinephrine transporters, although the psychoactive effects of cocaine have been primarily attributed to the effects on dopamine transmission (Rothman & Baumann, 2003). Decreasing striatal dopamine transmission in striatal regions attenuates cocaine-seeking. For example, rodents show a robust preference for an environment which they

associate with cocaine, but this can be blocked by administration of dopamine receptor antagonists (Cervo & Samanin, 1995; Pruitt, Bolanos, & McDougall, 1995). Additionally, most rats will work hard to self-administer cocaine, but this behavior is attenuated by striatal dopamine lesions (Belin & Everitt, 2008; Pettit, Ettenberg, Bloom, & Koob, 1984). The consistency of the aforementioned behavioral measures along with the involvement of dopamine in each mechanism makes conditioned place preference and self-administration paradigms commonly used pre-clinical models for assessing the reward.

Conditioned place preference is a form of Pavlovian conditioning and is a commonly used behavioral paradigm for assessing drug reward in rodents. In this paradigm, one area of a chamber is paired with a rewarding drug, such as cocaine, while the others are vehicle-paired and/or neutral over multiple conditioning sessions. If the animal chooses to spend significantly more time in the drug-associated versus neutral chambers, it is determined that they have formed a conditioned place preference. This paradigm is also sensitive in assessing aversive stimuli and less time spent in a drug-associated chamber would suggest a conditioned place aversion. One of the most unique and beneficial characteristics of this paradigm is that it assesses preference during a drug-free state. Additionally, once a condition place preference has been established, it remains robust for weeks and is highly resistant to extinction (de Wit & Stewart, 1981; Mueller, Perdikaris, & Stewart, 2002; Voigt, Herrold, & Napier, 2011). Arguably, this technique assesses the “liking” of a drug-paired chamber and includes the motivational component of “wanting”, because of the requirement of the animal to move into the preferred chamber. However, condition place preference is best

used in conjunction with other behavioral paradigms, such as self-administration, to elucidate the subjective effects of drugs.

Many testing schedules have been created to assess the variable components of drug self-administration including acquisition of drug seeking, drug-cue association, and extinction and reinstatement of drug-seeking. Perhaps the most translational, however, is the assessment of motivation, as it is a hallmark of the transition from casual drug use to addiction (Köpetz, Lejuez, Wiers, & Kruglanski, 2013). The progressive ratio schedule of reinforcement progressively increases the "cost" of drug by requiring an animal to nose-poke progressively more times for each additional infusion of drug. Once the cost outweighs the reward, or the point at which an animal will no longer makes enough nose-pokes, is considered their "breaking point". While all animals will eventually reach a "breaking point", specific variables have been identified to enhance motivation for females in particular, as discussed below.

Estradiol enhances female's vulnerability to addiction

Dopamine and estradiol

In females, estradiol rapidly increases striatal dopamine release after amphetamine administration (Becker, 1990a, 1990b). Estradiol binds to ERα and enhances dopamine release by inhibiting K^+-stimulated GABA release within the dorsal striatum, resulting in greater dopamine release due to a decrease of inhibition (Hu, Watson, Kennedy, & Becker, 2006; K. N. Schultz et al., 2009). In ventral stratum, ERβ enhances the effects of cocaine on electrically-stimulated dopamine release in gonadectomized females (Yoest et al., 2019a). Additionally, decreasing estradiol levels via ovariectomy in females reduces cocaine-induced dopamine levels but this can be

rescued with acute estradiol treatment (Cummings, Jagannathan, Jackson, & Becker, 2014).

Estradiol mediates drug-seeking in females

In the laboratory, escalation of drug taking can be measured by the rate at which rodents acquire self-administration of a drug after initial drug exposure. Exogenous estradiol is sufficient to enhance cocaine acquisition in ovariectomized females (Hu & Becker, 2008; Hu, Crombag, Robinson, & Becker, 2004; Lynch, Roth, Mickelberg, & Carroll, 2001). Estradiol does not facilitate or enhance acquisition of cocaine taking in males (Jackson, Robinson, & Becker, 2006). Sex differences in self-administration models are more robust in extended access paradigms versus short or intermittent access paradigms. This suggests that acquisition may be accelerated in females under certain conditions of drug accessibility (Algallal, Allain, Ndiaye, & Samaha, 2018; Roth & Carroll, 2004). The escalation of drug use is more difficult to pinpoint in humans, in part, due to changing environmental factors such as drug availability (Becker et al., 2012). Historically, drug availability has largely influenced women's use of opiates and psychostimulants as they were prescribed medications or marketing techniques to advance use of these drugs (Becker et al., 2012).

Under progressive ratio self-administration paradigms, when the "cost" of cocaine is high, females are more motivated to work for cocaine than are males (Kawa & Robinson, 2019). In intact female rodents, motivation for cocaine is modulated by circulating gonadal hormones and motivation is greatest during periods of the estrous cycle when estradiol is elevated (Becker & Hu, 2008; Becker & Koob, 2016; Roberts, Bennett, & Vickers, 1989). This idea is further supported by studies showing

ovariectomized adult females without estradiol replacement have lower motivation than those with estradiol (Hu & Becker, 2008; Perry, Westenbroek, & Becker, 2013). Together, these findings suggest that after initial acquisition of drug taking, females are more susceptible to escalate their motivation to attain drug and that this behavioral response of drug-seeking is enhanced by the presence of estradiol.

In rodent models, females in estrus also exhibited greater drug-primed reinstatement compared to females not in estrus and males (Kippin et al., 2005). Female rodents express signs of enhanced drug craving during estrus compared to non-estrus (Nicolas et al., 2019). In ovariectomized females, estradiol treatment potentiates reinstatement of drug-seeking (Becker & Hu, 2008; Doncheck et al., 2018; Larson & Carroll, 2007). Previous work also suggests that during drug-primed reinstatement, females who are in estrus display greater cocaine-seeking behavior than non-estrous females and males (Kerstetter et al., 2008). Further, females take longer to extinguish cocaine-seeking behaviors compared to males (Kerstetter et al., 2008). These studies suggest that estradiol plays a role in enhanced drug cravings in females, which may be contributing to the persistence of cocaine-seeking long into abstinence in females and related to the effects of estradiol on sensitization, as discussed above.

Over time, intake of psychostimulants by males also increases, but to a lesser degree than females. Furthermore, males intake does not appear to be regulated by testicular hormones (Hu et al., 2004). Males take longer to acquire a condition place preference for cocaine than females and require a higher dose of cocaine to acquire a preference (Zakharova, Wade, & Izenwasser, 2009). However, G Protein-coupled estradiol receptor-1 (GPER-1) has been implicated in being protective against

development of a preference for cocaine or opioids (Quigley & Becker, 2019; Sun et al., 2020). These findings indicate that estradiol is having different effects in males compared to females on neural processes related to addiction. In females, estradiol is enhancing vulnerability towards addiction-like behavioral while it is also possible that estradiol is acting in males to decrease vulnerability. The extent to which this is also true in humans needs to be investigated.

Estradiol Receptors

Estradiol mediates its effects through three receptors: ERα, ERβ, and GPER1. ERα was the first ER to be characterized (Jensen, 1962), and until the late 1990s many thought this single receptor mediated all of the functions of estradiol in an uncomplicated fashion. In 1996, researchers recognized ERβ as the second ER (Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996). GPER-1, previously known as GPR30, was recognized as an ER in the early 2000's (Thomas, Pang, Filardo, & Dong, 2005). Collectively these receptors mediate estradiol signaling using both rapid signaling and long-term transcription mediated responses. While rapid effects can occur anywhere between a few milliseconds to a few minutes, long-term effects take between a few hours and a few days (Farach-Carson & Davis, 2003).

Signaling mechanisms of estradiol receptors

The importance of understanding estradiol receptor-mediated signaling cannot be overstated. The outcome of treatment with estradiol will vary depending on the receptor's identity, location, function, and mechanism of action. ER signaling relies on four basic mechanisms: genomic, tethered, nongenomic (including caveolin-associated ERα and ERβ), and ligand-independent (Figure 1). Genomic and tethered mechanisms

occur within the nucleus, while non-genomic and ligand-independent mechanisms are extranuclear.

To mediate direct genomic effects, both ERα and ERβ can act as ligand-activated transcription factors, capable of directly affecting gene expression by interacting with regions of DNA called estrogen-response elements (ERE), as illustrated in Figure 1A (Marino, Galluzzo, & Ascenzi, 2006). As illustrated in Figure 1B, ERα and ERβ can also indirectly affect gene expression. In approximately 35% of the brain regions with ERs the EREs are not available for activation and the effect of estradiol is mediated by other intracellular signaling mechanisms (Marino et al., 2006; O'Lone, Frith, Karlsson, & Hansen, 2004; Vrtačnik, Ostanek, Mencej-Bedrač, & Marc, 2014). Additionally, through protein-protein interactions, ERα /ERβ signaling can enhance or suppress gene transcription independent of these EREs (Aranda & Pascual, 2001). Ligand-independent mechanisms that activate the ERE also work in the absence of ER agonists, as illustrated in Figure 1D.

In addition to their actions as separate entities, ERα & ERβ can combine to form a heterodimer with its own distinct effects on transcription (Cowley, Hoare, Mosselman, & Parker, 1997; Pettersson, Grandien, Kuiper, & Gustafsson, 1997). ERα and ERβ can function cooperatively in some cells and antagonistically in others (J Matthews & Gustafsson, 2003). For example, ERβ can directly modulate the activity of ERα by antagonizing ERα dependent transcription (Hall & McDonnell, 1999; Lindberg et al., 2003; Jason Matthews et al., 2006; Pettersson, Delaunay, & Gustafsson, 2000). Extranuclear ERs can regulate the recruitment of nuclear ERs, plasma membrane bound ERα signaling can affect the activity of nuclear ERα by stimulating

phosphorylation as well as facilitating its degradation (Bhatt, Xiao, Meng, & Katzenellenbogen, 2012; Reid et al., 2003). This mechanism is believed to explain the cyclic changes in the levels of ER-target gene expression (Reid et al., 2003). Activation of membrane bound ERs initiates signaling cascades that integrate at the level of the nucleus.

Rapid estradiol receptor signaling

Estradiol signaling can lead to rapid signaling cascades, long-term transcription effects, or both. Either mode of ER signaling can impact the connectivity and function of the brain. ERs associated with the membrane were initially discounted, but it is now recognized that membrane associated ERα and ERβ, along with GPER1, mediate important rapid effects of estradiol and some of these effects are implicated in addiction as discussed below.

Rapid ER signaling can be mediated by classical ERα and ERβ that are palmitoylated and bound to caveolin-1, a structural coat protein, and then trafficked to caveolae, which are invaginations of the plasma membrane that sequester many types of receptors and signaling molecules (Pedram, Razandi, Deschenes, & Levin, 2012; Razandi, Pedram, Merchenthaler, Greene, & Levin, 2004). Caveolin-1 facilitates anchoring these receptors to the caveolae, where estradiol can bind extracellularly and activate associated metabotropic glutamate receptors (mGluR) receptors (Luoma, Boulware, & Mermelstein, 2008; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). Multiple mGluRs are associated with ERα and ERβ in the hippocampus and dorsal striatum (Boulware et al., 2005; Boulware, Kordasiewicz, & Mermelstein, 2007; Grove-Strawser, Boulware, & Mermelstein, 2010). Rapid ER signaling via mGluRs is

implicated in the effects of estradiol on striatal dopamine release and cocaine self-administration (Martinez et al., 2016; Song, Yang, Peckham, & Becker, 2019).

Estradiol has been shown to rapidly enhance stimulated dopamine release and down-regulate D2 dopamine receptors in the dorsal striatum in vitro and in vivo (Bazzett & Becker, 1994; Becker, 1990b, 1990a; Becker & Ramirez, 1981; Cummings et al., 2014; Shams, Cossette, Shizgal, & Brake, 2018; Shams, Sanio, Quinlan, & Brake, 2016). Estradiol also rapidly regulates activity in the nucleus accumbens to affect post synaptic current in medium spiny neurons and stimulated dopamine release (Krentzel, Barrett, & Meitzen, 2019; Yoest et al., 2019a). These rapid effects of estradiol are implicated in acquisition of cocaine self-administration and motivation for cocaine in females, but not males as discussed above (Becker & Hu, 2008; Hu & Becker, 2008; Hu et al., 2004; Jackson et al., 2006).

In the hippocampus and associated circuitry, rapid ER signaling enhances social recognition, episodic memory, as well as object recognition and placement. The mechanism underlying this effect is believed to be the result of estradiol dependent rapid increases in dendritic spines (Frankfurt, Salas-Ramirez, Friedman, & Luine, 2011; Kim et al., 2019; Woolley, Weiland, McEwen, & Schwartzkroin, 1997). Whether similar ER-dependent changes in spine density is related to vulnerability to addiction remains speculative, but sex differences in cocaine effects on spine density and evoked neural activity in the nucleus accumbens core have been reported (Wissman, McCollum, Huang, Nikrodhanond, & Woolley, 2011).

Unlike, ERα and ERβ, GPER-1 is typically an extranuclear receptor embedded in several cell membranes, including the plasma membrane, endoplasmic reticulum, and

Golgi apparatus (Filardo et al., 2007; Funakoshi, Yanai, Shinoda, Kawano, & Mizukami, 2006; Kelly & Levin, 2001; Revankar et al., 2005; Sakamoto et al., 2007; Thomas et al., 2005; Waters et al., 2015). It can also translocate into the cytoplasm when activated (Funakoshi et al., 2006). GPER1 has been reported to enhance memory consolidation acting alone or in collaboration with ERα and ERβ (Hadjimarkou & Vasudevan, 2018; Kim et al., 2019). GPER1 may also attenuate vulnerability to addiction in male rodents (Quigley & Becker, 2019; Sun et al., 2020).

Localization of estradiol Receptors

Estradiol has been treated as though it acts uniformly throughout the brain on dopamine activity and addiction-related behaviors, but this is not the case (Cummings et al., 2014; Yoest, Cummings, & Becker, 2019b; Yoest et al., 2019a; Yoest, Quigley, & Becker, 2018). The types of estradiol receptors and where they are located in the brains of males and females provides potential pharmacological targets and neural locations for hormone-based treatments. Table 1 provides a comprehensive review of whole-brain ER distribution studies normalized such that ER densities can be compared among brain regions (Shughrue, Lane, & Merchenthaler, 1997a). Figure 2 A-C provides a visual comparison of ER densities, according to ER subtype in the rodent brain. Together, these tools provide a way to assess the contribution of ER subtypes within each brain region to addiction vulnerability.

Whole brain ER distribution studies have not found significant sex differences in ER expression, as can be seen in Table 1, however these findings are limited and insufficient to suggest no sex differences exist. For example, there are limited studies that include both males and females while looking ER expressions in the brain, and

fewer with the resolution to discern quantitative sex differences. Studies that examine individual brain areas do find some sex differences in ERs when assay conditions are enhanced to optimize expression or function for a particular brain region. In anatomical studies, it is not possible to discern mechanism of action of the receptors identified, so further research is needed to further determine the functional mechanisms mediating sex differences in many of the brain regions discussed below. Interestingly, while sex differences have not been investigated in all brain regions, there are sex differences in brain regions implicated in drug-taking and addiction.

In the ventral tegmental area, the number of dopamine cells that contained ERβ receptors was small, but males exhibited greater ERβ immunoreactivity in these neurons than females (Creutz & Kritzer, 2002). Intriguingly there were virtually no ERβ immunoreactive cells in the substantia nigra (Creutz & Kritzer, 2002). In the region of the lateral ventral tegmental area known as the parabrachial pigmented nucleus, ERβ-immunoreactivity is found in both dopamine and non-dopamine neurons and the proportion of dopamine neurons with ERβ was greater in males than in females, regardless of stage in estrous cycle, although females in diestrus had fewer ERβ positive neurons than those in proestrus (Creutz & Kritzer, 2002). The dopamine neurons in this brain region have been found to respond to low concentrations of ethanol and so the sex difference in ERβ dopamine neurons may be important for sex differences in addiction (Mrejeru, Martí-Prats, Avegno, Harrison, & Sulzer, 2015).

When examining ER expression in midbrain neurons that project to prefrontal cortex in male and female rats, different patterns were found. For males, none of the dopamine neurons labelled as projecting to the prefrontal cortical region contained ERα

or ERβ, while in females, some of the dopamine neurons labeled contained ERα, but not ERβ. This proportion of dopamine cells labeled in females was significantly different from males (Kritzer & Creutz, 2008). Thus, ERα and ERβ are strategically located to regulate motivational circuits differentially in males and females.

ERα receptor signaling plays a key role in the sexual differentiation of the mesolimbic reward pathway. ERα knockout animals show sex-specific differentiation patterns in the midbrain. ERα knockout female mice show increased levels of D1 dopamine receptor expression and dopamine receptor-interacting protein 78 (Drip78) mRNA levels (Küppers, Krust, Chambon, & Beyer, 2008). In contrast, ERα knockout males only showed decreased Drip78 mRNA levels (Küppers et al., 2008). With ERα knockout, both sexes showed reductions in midbrain expression of tyrosine hydroxylase (the enzyme catalyzing the rate limiting step for dopamine synthesis) and brain-derived neurotrophic factor (Küppers et al., 2008). Overexpression of ERα in dorsal striatum of female rats results in enhanced estradiol-induced motor activity and enhancement of the effect of estradiol to attenuate depolarization induced GABA release (K. N. Schultz et al., 2009). Electron microscope analysis of dorsal striatum finds ERα localized outside the nucleus of GABAergic neurons in female rats (Almey, Milner, & Brake, 2015). Thus, ERα is playing a role in striatal dopamine function indirectly mediated by rapid signaling through GABA neurons in females.

ERβ is also expressed in striatal regions, consistent with reports that ERβactivation regulates both the neurochemical and behavioral effects of drugs of abuse. In the dorsal striatum, ERβ activation upregulates D2 dopamine receptors (Le Saux, Morissette, & Di Paolo, 2006). An ERβ agonist induces immediate-early gene c-

fos expression in the nucleus accumbens, while an ERα agonist does not (Satta, Certa, He, & Lasek, 2018). ERβ's regions of action closely align with its alteration of the behavioral effects of a wide variety of drugs of abuse. Selective activation of ERβ enhances both amphetamine- and cocaine-induced CPP (Larson & Carroll, 2007; Satta et al., 2018; Silverman & Koenig, 2007). ERβ activation, but not ERα, results in enhanced stimulated dopamine release after cocaine in nucleus accumbens shell of females, but not males (Yoest et al., 2019a). Finally, ERβ receptor signaling, but not ERα, mediates estradiol's effect on cocaine-induced reinstatement of extinguished cocaine-seeking behavior in OVX rats (Larson & Carroll, 2007).

In the cortex, there is a greater expression of GPER1 than in ERα and ERβ, pointing to a role for GPER1 in higher order cognitive functions (Table 1). Importantly, while expression patterns differ, as can be seen in Figure 2, they are also strongly overlapping giving the potential for these receptor mechanisms to interact. Recently, GPER1 has been identified as the first estradiol receptor to modulate the preference for rewarding stimuli in males. A decrease of GPER1 in the CNS, via gene knockout, increases the acquisition of conditioned place preference for morphine in males (Sun et al., 2020). GPER1 has also been implicated in enhancing memory consolidation, via enhanced dendritic spine density in the CA1 region of the hippocampus, in female mice (Gabor, Lymer, Phan, & Choleris, 2015). Together, these findings suggest that GPER1 activation could be enhancing memory for environmental stimuli/cues related to a drug-induced state and causing a more rapid formation of conditioned place preference in females, while decreasing these associations in males.

Summary of Dissertation Experiments

The presence of estradiol enhances the rewarding properties of drugs of abuse and drug-seeking for females but the effects of estradiol in males is understudied. Further, few studies have investigated the contribution of specific estradiol receptor subtypes, ERα, ERβ or GPER1, on drug seeking in either sex. This gap may be contributing to the lack of effect of estradiol reported in males, thus far.

Chapter II: Activation of G-protein coupled receptor 1 in the dorsolateral striatum attenuates preference for cocaine and saccharin in male but not female rats

The goal of chapter II was to investigate how manipulation of estradiol receptors alters the acquisition of cocaine conditioned place preference. After finding that pharmacological activation of GPER1 in the dorsolateral striatum (DLS) is sufficient to inhibit cocaine conditioned place preference in males, I investigated the effects of intra-DLS GPER1 activation on acquisition of saccharin. Similar to the effects observed with cocaine, preference for saccharin was abolished in males. In the final experiment of this chapter, I determined that there are not sex differences in mRNA levels of ERα, ERβ or GPER1 in the dorsal striatum.

Chapter III: Activation of G-protein coupled estradiol receptor 1 in the dorsolateral striatum enhances motivation for cocaine and drug-induced reinstatement in female rats

In chapter III, I sought to determine how GPER1 activation intra-DLS might influence motivation for cocaine in males and female. First, I assessed how intra-DLS GPER1 activation alters males' and females' motivation to attain cocaine. Next, I extinguished drug-seeking in all animals prior to testing whether their drug-induced reinstatement of drug-seeking was altered by intra-DLS GPER1 activation. I reported

that activation of GPER1 not only enhances females' motivation for cocaine, but significantly enhances their reinstatement of drug-seeking.

Chapter IV: Alterations of striatal c-Fos immunoreactivity induced by G-protein coupled estradiol receptor 1 activation

The goal of chapter IV was to gain insight on how GPER1 activation intra-DLS could be mitigating the rewarding properties of cocaine for males. To that end, I investigated patters of cocaine-induced neuronal activation in reward-regions of the brain in addition to patterns of activation that are altered by pharmacological manipulation of GPER1.

			ERα		ERβ		GPER1	
			mRNA	Protein	mRNA	Protein	mRNA	Protein
Cerebral Cortex (PFC; F, P, T, O)		F	–(Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998)	+(Mitra et al., 2003)	+(Laflamme et al., 1998)	[x]; ++,++,++,++(Shughrue & Merchenthaler , 2001)	+++(Morissette et al., 2008)	[x]; +++ (Hazell et al., 2009)
		M	–(Laflamme et al., 1998)		+(Laflamme et al., 1998)		+++(Morissette et al., 2008)	[x]; +++ (Hazell et al., 2009)
Hippocampus (CA1, CA2, CA3, DG)		F	+, +, ++, [x](Laflamme et al., 1998)	+,+,+,+(Mitra et al., 2003)	+,–,+, [x](Laflamme et al., 1998)	–, +, +, ++(Shughrue & Merchenthaler , 2001)	++(Morissette et al., 2008)	++,++,++,+++ (Hazell et al., 2009)
		M	+, +, ++, [x](Laflamme et al., 1998)		+,–,+, [x](Laflamme et al., 1998)		++(Morissette et al., 2008)	++,++,++,+++ (Hazell et al., 2009)
Striatum		F		+(Mitra et al., 2003)		++++(Shughrue & Merchenthaler , 2001)		+(f) (Hazell et al., 2009)
		M						+(f) (Hazell et al., 2009)
BNST		F	++++(Shughrue, Lane, & Merchenthaler , 1997b); ++(Laflamme et al., 1998)	+++(Mitra et al., 2003)	++++(Shughrue et al., 1997b); +(Laflamme et al., 1998)	++++(Shughrue & Merchenthaler , 2001)		+ (Hazell et al., 2009)
		M	++(Laflamme et al., 1998)		+(Laflamme et al., 1998)			+ (Hazell et al., 2009)
Hypothalamus	PVN, SON	F	–, –(Shughrue et al., 1997b)	+,+(Mitra et al., 2003)	+, +++(Shughrue et al., 1997b)	++++(Shughrue & Merchenthaler , 2001)		++++,++++ (Hazell et al., 2009)
		M						++++,++++ (Hazell et al., 2009)
	Preoptic Area (Medial, Lateral, Periventricular)	F	++++, ++, +++(Shughrue et al., 1997b); ++++, ++, ++(Laflamme et al., 1998)	+++,+,++(Mitra et al., 2003)	++++,++,+++(Shughrue et al., 1997b); +++,– ,++(Laflamme et al., 1998)	++, ++, +++(Shughrue & Merchenthaler , 2001)		+++,+,+++ (Hazell et al., 2009)

	M	++++, ++, ++(Laflamme et al., 1998)		+++,–,++(Laflamme et al., 1998)			+++,+,+++ (Hazell et al., 2009)
Ventromedial Hypothalamus (Dorsomedial., Ventrolateral)	F	++, ++++(Laflamme et al., 1998)	+, ++++(Mitra et al., 2003)	–, ++(Laflamme et al., 1998)	++(Shughrue & Merchenthaler, 2001)		++++,++++ (Hazell et al., 2009)
	M	++, ++++(Laflamme et al., 1998)		–, ++(Laflamme et al., 1998)			++++,++++ (Hazell et al., 2009)
Medial Amygdala	F	++++(Shughrue et al., 1997b); ++(Laflamme et al., 1998)	+++(Mitra et al., 2003)	+++(Shughrue et al., 1997b); ++(Laflamme et al., 1998)	++++(Shughrue & Merchenthaler, 2001)		+ (Hazell et al., 2009)
	M	+++(Laflamme et al., 1998)		++(Laflamme et al., 1998)			+ (Hazell et al., 2009)
VTA	F	+(Shughrue et al., 1997b)	–(Mitra et al., 2003)	++(Shughrue et al., 1997b)	++(Shughrue & Merchenthaler, 2001)		– (Hazell et al., 2009)
	M						– (Hazell et al., 2009)
Periaqueductal Grey	F	+(Shughrue et al., 1997b), +++(Laflamme et al., 1998)	+++(Mitra et al., 2003)	+(Shughrue et al., 1997b); +(Laflamme et al., 1998)	++(Shughrue & Merchenthaler, 2001)		++ (Hazell et al., 2009)
	M	+++(Laflamme et al., 1998)		+(Laflamme et al., 1998)			++ (Hazell et al., 2009)
Substantia Nigra	F	++(Laflamme et al., 1998)	+(Mitra et al., 2003)	+(Laflamme et al., 1998)	+(Shughrue & Merchenthaler, 2001)		+++ (Hazell et al., 2009)
	M	++(Laflamme et al., 1998)		+(Laflamme et al., 1998)			+++ (Hazell et al., 2009)
Locus Coeruleus	F	+(Shughrue et al., 1997b); +++(Laflamme et al., 1998)	+(Mitra et al., 2003)	+(Shughrue et al., 1997b); –(Laflamme et al., 1998)	+(Shughrue & Merchenthaler, 2001)		++++ (Hazell et al., 2009)
	M	+++(Laflamme et al., 1998)		–(Laflamme et al., 1998)			++++ (Hazell et al., 2009)
Parabrachial (Medial, Lateral)	F	–, +(Shughrue et al., 1997b)	+,++(Mitra et al., 2003)	+,+(Shughrue et al., 1997b)	++,++(Shughrue & Merchenthaler, 2001)		–,+++ (Hazell et al., 2009)
	M						–,+++ (Hazell et al., 2009)

Cerebellum (purkinje cells, granulosa cells)	F	–(Laflamme et al., 1998)	–(Mitra et al., 2003)	++(Laflamme et al., 1998)	+, +++(Shughrue & Merchenthaler, 2001)		++++ (Hazell et al., 2009)
	M	–(Laflamme et al., 1998)		++(Laflamme et al., 1998)			++++ (Hazell et al., 2009)
Pituitary Gland (Ante., Post., Int.)	F		++++, [x], +++(Mitchner, Garlick, & Ben-Jonathan, 1998)		++++, [x], +++(Mitchner et al., 1998)	+, –(Hazell et al., 2009)	+, ++++(f), +++ (Hazell et al., 2009)
	M					+, –(Hazell et al., 2009)	+, ++++(f), +++ (Hazell et al., 2009)

Table 1 Distribution of estradiol receptors and corresponding mRNA transcript in the CNS of mice and rats.

For all brain regions, Mitra et al. (2003) and Hazell et al. (2009) refer to data from mice. All other references refer to data from rats, including Hazell et al. (2009) for the pituitary gland. Data have been normalized to fit the following scale (Shughrue et al., 1997): , no signal; +, low signal; ++, moderate signal; +++, intense signal; ++++, very intense signal. Comma separations correspond to respective subregions; when no commas are used, the whole region is implicated; [x] indicates a subregion not specifically noted by the source's data.

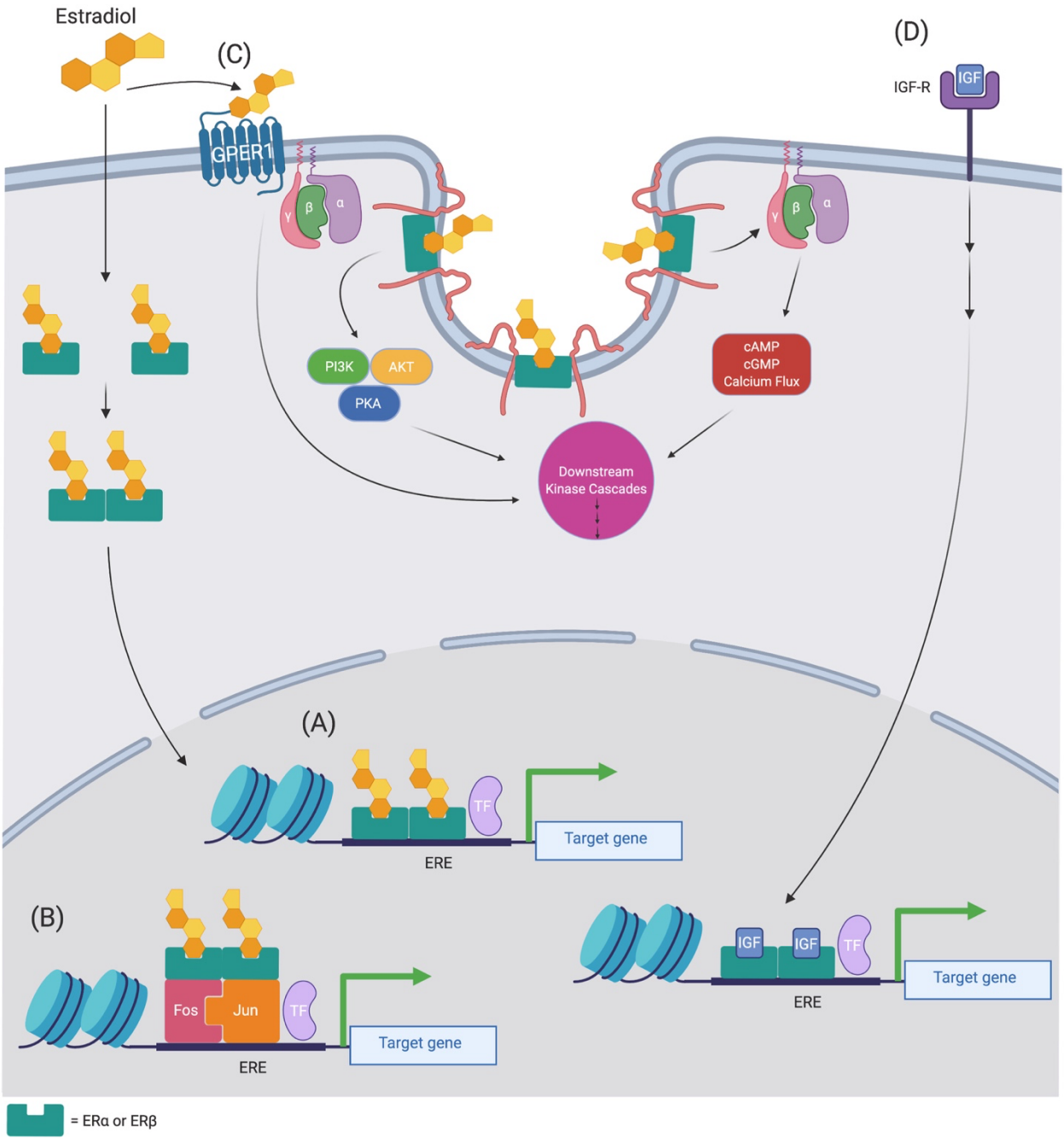
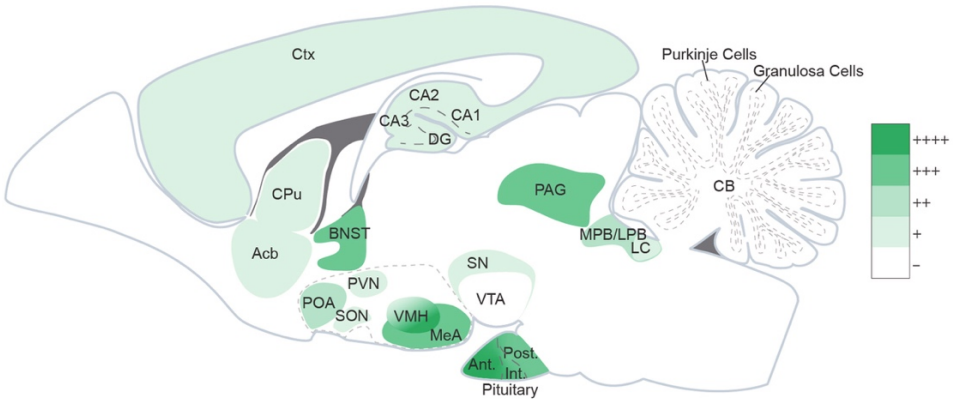

Estradiol
(C)
GPER1
γ
β
α
PI3K
AKT
PKA
(D)
IGF-R
IGF
cAMP
cGMP
Calcium Flux
Downstream
Kinase Cascades
(A)
TF
Target gene
ERE
(B)
Fos
Jun
TF
Target gene
ERE
IGF
IGF
TF
Target gene
ERE
= ERα or ERβ

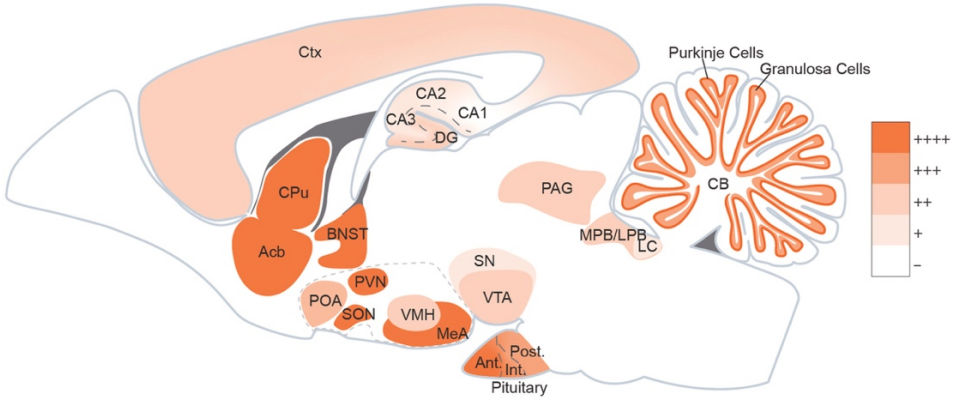
Figure 1 The four core pathways of estradiol receptor action include: genomic, tethered, non-genomic, and ligand-independent.

(A) The direct most direct mechanism of ER action mediates gene transcription at ERE (estradiol response element) sites. When estradiol encounters a cell, some will pass through the plasma membrane and into the nucleus. ERs exist as monomers in multiprotein inhibitory complexes until activated by estradiol (Klinge et al., 1997). This activation causes a conformational change that allows ERs to dimerize and migrate to the EREs (Ogawa et al., 1998). Interaction between this estradiol/ERs complex, steroid receptor coactivators (SRC), and RNA polymerase II enhances the transcription of downstream targets (Hall et al., 2001; Björnström and Sjöberg, 2005; Kininis and Kraus, 2008). (B) Activated ERs do not always directly interact with EREs but rather "tether" to transcription factors such as specificity protein (Sp-1) or activating protein-1 (AP-1), to form protein-protein complexes that alter transcription (Safe and Kim, 2008; Paech et al., 1997). In the absence of an activated ER, Sp-1 and AP-1 do not influence transcription (Ahlbory-Dieker et al., 2009; Hewitt et al., 2014). (C) Non-genomic actions are responsible for rapid estradiol mediated signaling via extranuclear ERs bound to different membranes in the cell (Pietras and Szego, 1977). Caveolae are populated by g-protein subunits and upon activation, these proteins cause signaling cascades that ultimately produce cAMP, cGMP, calcium flux, and protein-kinase activation (Marino et al., 2006; Levin and Hammes, 2016; Lösel and Wehling, 2003). There are four major protein-kinase cascades: phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT, and cAMP/protein kinase A (PKA)(Marino et al., 2006). GPER-1 is a unique ER in the sense that it can initiate these signaling cascades on its own. (D) Ligand-independent mechanisms work in the absence of estradiol. Upon activation, growth factor receptors (GFRs) on the plasma membrane initiate signaling cascades, as described above (Bennesch and Picard, 2015). This results in the activation of nuclear ERs by either phosphorylating the receptor itself or stimulating the recruitment of steroid receptor coactivators (SRCs).

ERα



ERβ



GPER1

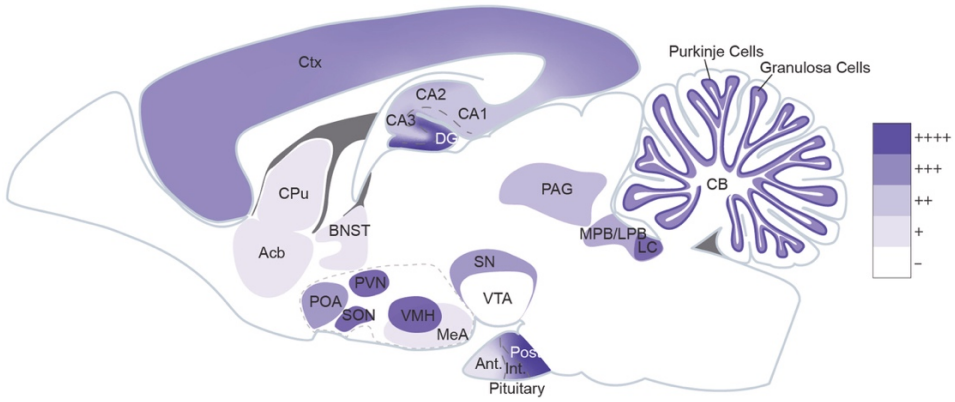


Figure 2 Graphical representation ERα, ERβ, and GPER1 localization in the CNS.

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Chapter II
Activation of G-protein Coupled Estradiol Receptor 1 in the Dorsolateral Striatum Attenuates Preference for Cocaine and Saccharin in Male but Not Female Rats

Abstract

There are sex differences in the response to psychomotor stimulants, where females exhibit a greater response than males, due to the presence of the gonadal hormone estradiol. Extensive research has shown that estradiol enhances drug-seeking and the rewarding properties of cocaine for females. The role of estradiol in male drug-seeking, however, is not well understood. The current study investigated pharmacological manipulation of estradiol receptors in the dorsolateral striatum (DLS) on preference for cocaine in gonad-intact male and female rats. In males, activation of G-protein coupled estradiol receptor 1 (GPER1), via administration of ICI 182,780 or G1, attenuated conditioned place preference for 10mg/kg cocaine, while inhibition of GPER1, via G15, enhanced preference at a 5mg/kg cocaine dose. Similarly, GPER1 activation, via G1, prevented males from forming a preference for 0.1% saccharin (SACC) versus plain water. Surprisingly, activation of GPER1 did not alter preference for cocaine or SACC in females. These studies also examined the quantity of estradiol receptor mRNA in the dorsal striatum, using qPCR. No sex differences in relative mRNA expression of ERα, ERβ, and GPER1 were observed. However, there was greater GPER1 mRNA, relative to ERα and ERβ, in both males and females. The results presented here indicate that estradiol, acting via GPER1, may be protective against drug preference in male rats.

Introduction

More men report abusing cocaine and qualify for cocaine use disorder, however, cocaine use in women is gradually increasing and there is growing evidence to suggest that women are more vulnerable to addiction [1]. The time of first drug use to admission for addiction treatment is typically shorter for women than men [2]. When entering treatment, women also present with greater social, behavioral, and psychological symptoms related to substance use disorder, despite having abused drugs for a shorter period of time [3,4]. These women are also taking greater amount of drug when entering treatment and report experiencing enhanced cravings compared to their male counterparts [5,6]. 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 that enhances drug-taking in females [7,8].

Research into the biological bases for sex differences in cocaine addiction has focused on how estradiol alters the rewarding properties of cocaine in females. Clinical models have found that when estradiol levels are high, women report an enhanced euphoria or “high” when abusing smoked cocaine [9]. Similarly, female rats show enhanced cocaine craving and motivation for cocaine when estradiol levels are highest [10–13]. While these data suggest that estradiol plays a major role in facilitating motivation and other behaviors in females, the role of estradiol in modulating males’ addiction-like behaviors should also be understood. Testosterone produced by the male testes is aromatized to estradiol in the brain and periphery to act on estradiol receptors in the male brain.

Estradiol acts by binding to receptors ERα, ERβ, and GPER1. The cellular location of traditional estradiol receptors α and β are either in the cell nucleus, or after palmitoylation, on the extracellular membrane in association with caveolin proteins [14–16]. GPER1 activates intracellular signaling cascades mediated by cAMP, ERK, and PI3K and localized on the plasma membrane and in the endoplasmic reticulum [17]. In females, ERα and GPER1 in the dorsal striatum are localized to GABAergic medium spiny neurons with recurrent collaterals onto dopamine terminals, as well as cholinergic interneurons and glia [18,19]. The localization of ERα and GPER1 in the dorsal striatum of males remains unknown.

For males and females alike, the rewarding effects of cocaine are attributed to the drug's direct effects on dopamine reuptake in the striatum [20,21]. However, there are sex differences in the effects of cocaine on increases in extracellular dopamine and receptor binding that are driven by estradiol in the dorsal striatum. In females, estradiol binding to GABAergic neurons decreases GABA release and this disinhibits dopamine terminals and ultimately increases stimulated dopamine release locally [16,22]. This estradiol-induced increase in dopamine release is associated with an enhancement of the effects of cocaine and other psychostimulants on drug-induced behaviors, such as behavioral sensitization [23–26]. Previous research has not found that estradiol has similar effects on cocaine-induced increases in dopamine in males [23]. Finally, the effects of estradiol in dorsolateral striatum (DLS) are mediated by mGluR signaling in females [24]

There is extensive support for estradiol in mediating drug abuse liability in females but a lack of attention to understanding the role of estradiol in males. There is recent evidence from gene knockout studies, however, that GPER1 could be playing a

modulatory role in the preference for morphine in males [27]. Given that previous research has highly implicated the DLS as an important region for studying the effects of estradiol on addiction-like behaviors in females, the current set of studies were deigned to investigate how intra-DLS GPER1 modulates preference for rewarding stimuli in males as well as females. A cocaine conditioned place preference (CPP) and saccharin (SACC) two-bottle choice behavioral paradigms were used to assess how GPER1 activation or inhibition alters preference for rewarding stimuli. Finally, the current study also used qPCR to determine relative mRNA levels of ERs in the dorsal striatum of both males and females.

Materials and Methods

Animals

A total of 62 male and 46 female gonad-intact Sprague-Dawley rats were used in the current set of experiments, as detailed in Figure 3 A. Animals were ordered from Charles River Breeding Laboratory (Portage, MI, USA) and were approximately 75 days old on arrival. Animals were maintained on a 14:10 light/dark cycle in a temperature-controlled climate of 72°F ± 2°F, in ventilated laboratory cages. 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). Animals were initially housed in same-sex pairs until undergoing surgery, after which they were subsequently housed individually. All animals were weighed daily to determine good health and at this time, females were also vaginally lavaged to determine stage of estrus. 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 Use and Care of Animals Committee.

Stereotaxic Surgery and Drug Preparation

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 were 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, 33-gauge solid stylets were inserted into the 26-gauge hollow guide cannula that were fixed on animals' skull. These stylets were flush with the bottom of the guide cannula and did not protrude into the brain. Treatment conditions were randomly assigned to animals prior to behavioral testing. Control animals received 100% cholesterol (CHOL) and experimental animals received either 10% ICI (ERα/ERβ antagonist; GPER1 agonist), G1 (agonist targeting GPER1) or G15 (antagonist targeting GPER1) dissolved in Cholesterol (Control) via stylets, which protruded from the guide cannula by 1mm and delivered treatment directly into the DLS. Treatment stylets were prepared as previously described [28]. In order to insert stylets, rats were briefly anesthetized with 5% isoflurane.

Hollow guides and interlocking treatment stylets were manufactured by and purchase from P1 Technologies. Drugs were obtained from the following sources: ICI182,780 (ICI) (Santa Cruz Biotechnology, purity ≥ 98%); G1 (Cayman Chemical, purity ≥ 98%); G15 (Cayman Chemical, purity ≥ 95%); Cholesterol (Santa Cruz Biotechnology, purity ≥ 92%)

Conditioned Place Preference (CPP)

Animals were tested on a CPP paradigm that took place over 10 consecutive days, as illustrated in Figure 4 A. The CPP apparatus consisted of two side chambers (15.5 inches x 12 inches) and a center neutral chamber (15.5 inches x 7.5 inches). On day 1 (pre-test), rats were placed in the novel chamber and were allowed to move freely between all compartments for 30 minutes. Immediately following pre-test session, treatment stylets were inserted. 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 habituation in the center neutral chamber. Animals were then 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 to each stimulus (drug or vehicle) 4 times each, every other day. Which treatment animals received first was counterbalanced. On day 10 (Test), rats were placed in the three-compartment chamber and were again allowed to move freely between all compartments for 30 minutes.

The side in which animals spent the most time during pre-test was treated as their “preferred chamber”, which differed for each animal. A biased design was utilized,

so that each individual animal's initially preferred chamber was paired with saline, and their initially un-preferred chamber was paired with cocaine for conditioning. ANYMAZE tracking software (Stoelting Co., Wood Dale, IL 60191) was utilized to track the amount of time spent in each chamber.

Either a 5mg/kg or 10mg/kg cocaine dose was utilized during conditioning in order to be able to test both an increase and decrease in cocaine CPP after ER manipulation. For example, both males and females acquire cocaine CPP at a 10mg/kg conditioning dose therefore we utilized that dose when investigating a potential decrease in CPP (i.e., ICI and G1). When investigating whether there was an increase cocaine CPP in males, we used a dose that did not generally produce a CPP, 5mg/kg, which allowed us to identify a potential increase in CPP (i.e., G15) without a ceiling effect. This dose of cocaine did produce CPP for cocaine in females (data not shown), so we did not test the effects of G15 in females with 5mg/kg cocaine.

Two Bottle Choice Experiment (SACC versus H_2O)

Animals were tested on a two-bottle choice paradigm that took place over 12 consecutive days, as illustrated in Figure 3 B. Briefly, on days 1-4, two bottles both containing water were available on each animal's home cage to determine that were was no significant difference between total liquid intake between animals. During days 5-12, one bottle contained water and the second bottle contained 0.1% saccharin (Sigma-Aldrich; purity ≥ 92%) dissolved in water. Placement of the bottle (left versus right) was switched daily to account for a potential side of cage preference. On day 5, four hours before the SACC bottle was introduced, stylets containing either 10% G1 in cholesterol or cholesterol alone were inserted and remained in place for the duration of

the experiment. Once daily, one hour prior to the start of dark cycle, bottles were removed from home cages, weighed, and refilled.

Euthanasia and Tissue Preparation

Animals received 0.5 ml of Sodium Pentobarbital (i.p). Once the animal was fully sedated, it was perfused transcardially with 0.1M phosphate buffered saline followed by 4% paraformaldehyde. The brain of each rat was also dissected and post-fixed in 4% paraformaldehyde 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 guide cannulae placements, depicted in Figure 3, by an observer blind to experimental conditions. Only animals that had accurate cannulae placements are shown and were included in the final analysis. For animals in the H_2O versus SACC experiment, females' ovaries and uterus and males' testes and vas deferens were dissected and weighed after the animal was perfused as a proxy to determine if G1 treatment in the brain affected peripheral gonadal tissues.

Quantitative Polymerase Chain Reaction (qPCR)

Naïve gonad-intact male and female rats were given 0.5 ml of FatalPlus (i.p.; 195 mg sodium pentobarbital; Vortech Pharmaceuticals, Ltd; Dearborn, MI). Once the animal was fully anesthetized the brain was rapidly removed and placed into ice-cold saline. The dorsal striatum 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). Primers (not designed to determine specific splice variants, but to detect all variants) were purchased from Qiagen for: ERα (Cat. No. QT00386925, Qiagen Primer; reference sequence NM_012689), ERβ (Cat. No. QT00190113, Qiagen Primer; reference sequence NM_012754), and GPER1 (Cat. No. QT00376943, Qiagen Primer; reference sequence NM_133573). These genes were compared against the housekeeping gene HPRT1 (Cat No. QT00199640, Qiagen Primer) and relative gene expression was quantified using the 2^ddCT method. Samples were run in triplicates at the Biomedical Research Core Facilities at the University of Michigan (https://brcf.medicine.umich.edu/cores/advanced-genomics/technologies/real-time-pcr/).

Statistical Analysis

CPP data were analyzed using 2-way ANOVAs. In the case of a significant interaction, a Bonferroni correction was used for multiple comparisons. For males and females independently, CPP data were analyzed by time spent in the drug-paired chamber (pre-test versus test) between treatment conditions, within each sex. Behavioral testing for males and females was not done simultaneously therefore, we did not compare them statistically.

Daily preference of 0.1% SACC versus water was calculated as a percentage: (0.1% SACC consumed/(0.1%SACC + Water consumed))*100. An unpaired t-test was performed to identify treatment group differences in preference score. Unpaired t-tests

were performed to determine if gonad weights were different between G1 or CHOL treated males or females.

Relative mRNA expression of ERα, ERβ, and GPER1 was analyzed between sexes. Unpaired t tests were performed to identify sex differences in ERβ and GPER1 expression within the dorsal striatum. Nonparametric testing was conducted for ERα because data were not normally distributed for males.

All statistical analyses were performed using GraphPad Prism v8.0 and IBM SPSS Statistics v27.0. All data sets were tested for normality and equal variance between groups. Effect sizes are reported as partial eta ($n^{2)}$ or partial eta-squared (n^2p) for F-tests and Cohen's d (d) for t-tests. The threshold for significance in all experiments was set to $p<0.05$ and sample sizes per experiment were determined based on pilot studies. Animals were excluded from statistical analyses if the placements of the guide cannula were off target in both the CPP and SACC versus H_2O experiments (< 5% of total animals were excluded).

Results

Effects of ER manipulation on cocaine CPP in males

The effects of ER manipulation on cocaine CPP in males, as measured by time spent in the drug-paired chamber, is shown in Figure 7 A-C. Administration of ICI, a nonselective ERα/β antagonist and GPER1 agonist, attenuated males' preference for 10mg/kg cocaine (Figure 5 A). A two-way repeated measures ANOVA revealed a treatment x test session interaction ($F_{(1,15)} = 5.758$; $p = 0.0299$, $n^2p = 0.277$). A Bonferroni multiple comparisons test revealed that CHOL treated animals increased

time spent in the cocaine-paired chamber (4.745 ± 0.974, p = 0.0004) but ICI treated males did not (0.482 ± 1.033, p > 0.9999).

The goal of our follow up study was to differentiate between the effects of ICI on inhibition of ERα and ERβ versus activation of GPER1. We did this by using G1, a selective GPER1 agonist. Figure 5 B represents males treated with G1 compared to CHOL. A two-way repeated measures ANOVA revealed that G1 treatment replicated the behavioral effect of ICI treatment on cocaine CPP; there was a significant interaction between test session and treatment ($F_{(1,15)} = 7.429$; p = 0.016, $n^2p = 0.331$) and multiple comparisons revealed that at 10mg/kg cocaine, G1 treated males did not acquire a CPP for cocaine (1.272 ± 0.972, p = 0.3804), but CHOL animals did (4.745 ± 0.8741, p = 0.0001).

We further investigated whether administration of the GPER1 antagonist, G15, could cause enhanced preference for cocaine in males. Illustrated in Figure 5 C, at a 5mg/kg conditioning dose, CHOL treated males did not show a CPP for cocaine however, G15 treated males did. A two-way repeated measures ANOVA revealed a significant interaction between test session and treatment ($F_{(1,15)} = 8.194$; p = 0.0119, $n^2p = 0.353$). A Bonferroni multiple comparisons test revealed that males treated with G15 spent more time in the drug-paired chamber after conditioning while CHOL treated males did not (1.591 ± 0.6143, p = 0.0406; 0.826 ± 0.579, p = 0.3182; respectively).

Effects of ER manipulation on cocaine CPP in females

The effects of ER manipulation on cocaine CPP in females are shown in Figure 5 D-E. When cocaine CPP was determined in females treated with CHOL, ICI, or G1, all groups formed a preference for 10mg/kg cocaine. A two-way repeated measures

ANOVA was performed to compare test sessions by treatment. Figure 5 D represents CHOL and ICI treated females, where there was a main effect of test session ($F_{(1,16)}$ = 13.99; p = 0.018, n^2p = 0.467). There was no main effect of treatment however, and no interaction between test session and treatment. Similar results are shown in Figure 5 E, which represents CHOL and G1 treated females, where there was only a main effect of test session ($F_{(1,16)}$ = 20.05; p = 0.0004, n^2p = 0.556).

Effects of intra DLS ER manipulation on saccharin preference in males and females

The effects of the GPER1 agonist, G1, on preference for 0.1% SACC versus water is illustrated in Figure 5 D. An unpaired t-test comparing preference scores between CHOL and G1 treated males revealed significant group differences ($t_{(10)}$ = 2.589; p = 0.0270, d = 1.495) in preference scores. There were no differences in total water intake on days 1-4, prior to treatment. There were also no significant differences in total liquid consumed on days 5-12 between treatment groups.

Consistent with the effects of G1 on cocaine CPP, there was no effect of G1 on females' preference for 0.1% SACC over water, shown in Figure 6 C. An unpaired t-test was used to reveal no significant group differences ($t_{(10)}$ = 0.7998; p = 0.4424, d = 0.462). Both G1 and CHOL groups had equal variance and data were normally distributed. There were no differences in total liquid consumption on days 1-4, prior to treatment or during treatment on days 5-12.

Effects of intra DLS ER manipulation on gonad weights in males and females

There was no effect of G1 treatment intra-DLS on gonad weight in either males or females. In females, this included uterus weight ($t_{(10)}$ = 0.4571; p = 0.6574, d = 0.263) and ovary weights ($t_{(10)}$ = 1.892; p = 0.0878. d = 1.09). For males, this included

testes ($t_{(10)} = 0.1270$; $p = 0.9014$, $d = 0.073$) and vas deferens weights ($t_{(10)} = 1.625$; $p = 0.1352$, $d = 0.938$).

Relative mRNA expression of ERα, Erβ, and GPER1 in the dorsal striatum of males and females

As shown in Figure 7 A-C, there were no sex differences in relative mRNA expression of ERs in the dorsal striatum. For ERα, male data points violated normality testing, therefore an unpaired nonparametric Mann-Whitney U test was performed to identify group differences. No sex differences in ERα expression were found ($U = 19$; $p = 0.1949$, $n = 0.142$). Unpaired t tests revealed no group differences for ERβ ($t_{(14)} = 0.3334$; $p = 0.7438$, $d = 0.166$), or GPER1($t_{(14)} = 0.7504$; $p = 0.4654$, $d = 0.374$).

The expression of GPER1 mRNA was greater than ERα and Erβ mRNA relative to the HRPT housekeeping gene, as illustrated in Figure 7 D. A significant one-way ANOVA determined differences between ER subtypes ($F_{(2,45)} = 180.3$; $p < 0.0001$, $n^2 = 0.889$). A Bonferroni multiple comparisons test determined a significant difference in relative expression of GPER1 versus ERα ($p < 0.0001$) and GPER1 versus ERβ ($p < 0.0001$) but no difference between ERα and ERβ ($p > 0.9999$).

Discussion

Our study is the first to report that GPER1 activation in the DLS modulates preference formation for cocaine in male rats. We have shown that activation of GPER1 in the DLS is sufficient to attenuate cocaine CPP, while inhibition of GPER1 receptors produces a significant preference for cocaine at a dose that does not usually induce CPP in males. We also identified the relative mRNA expression of ERs in the dorsal striatum and found no sex differences, indicating that the sex-specific effect of GPER1

activation/inhibition is not due to an overall sex difference in the expression of receptors within this brain region.

Previous work in male rats found that animals that formed a strong taste aversion to amphetamine, relative to animals that formed a low taste aversion, also formed a stronger place preference for amphetamine, suggesting a common mechanism mediates both effects [29]. Our findings that GPER1 activation inhibits preference for cocaine in males was replicable with an alternative reward, 0.1% SACC, which was preferred in control males as well as both experimental and control females. These animals still drank comparable amounts of liquid and consumed equitable amounts of chow compared to controls, which suggests that GPER1 activation does not cause an overall malaise in male animals. Instead, we hypothesize that GPER1 activation could be altering the learned rewarding effects of cocaine and SACC. In both experiments, the rewarding stimuli were introduced after G1 was administered into the DLS. Future experiments are needed to determine whether GPER1 activation would decrease the rewarding properties of cocaine or SACC after a preference has been established, or if the effect of GPER1 is limited to the initial establishment of a preference.

We initially hypothesized that the pharmacological manipulation of ERs in the DLS of females would alter their cocaine CPP, based on previous findings that estradiol regulates the rewarding properties of cocaine in females [30]. We predicted that administering ICI, an ERα and ERβ antagonist would inhibit CPP formation, but our results do not support this. Both ICI and G1 attenuated cocaine CPP in males, but not females. Since ICI is an ERα and ERβ antagonist, and a GPER1 agonist, this suggests that it was the GPER1 agonist action that attenuated CPP in males. We postulate that

the effects of ICI in females were seen because it is also an agonist for GPER1 and that we would have needed to give an antagonist all three receptors to inhibit cocaine preference formation in females. We also recognize that a significant limitation of this study is the lack of varied doses, as we only administered 10% drug:vehicle in both sexes. Although this was a sufficient dose in males, it could be that females need a higher or lower dose to cause a change in behavior.

Females show enhanced rotational behavior and sensitization after cocaine exposure compared to males and this effect is mediated by ERα [25,31]. Work in dissociated medium spiny neurons from dorsal striatum of females has shown estradiol decreases L-type calcium current, which implicates ERβ [32,33]. In neurons from males, the response to estradiol was significantly less [34]. Finally, estradiol treatment reduces GABA release, and overexpression of ERα in the dorsal striatum also enhances the inhibitory effects of estradiol on GABA release [31,35]. Thus, ERα and ERβ are implicated in the effects of estradiol on striatal function in females.

Our study used qPCR to explore relative RNA expression of ERα, ERβ, and GPER1 in the dorsal striatum and we did not find any sex differences. These findings are consistent with recent evidence supporting no sex differences in protein levels of ERα or GPER1 in the dorsal striatum of adult males and females [36]. Our study did determine that GPER1 mRNA levels are greater than ERα and ERβ, which do not differ from one another.

We did not differentiate the medial versus lateral subsections of the dorsal striatum, and distribution of ERs within the dorsal striatum could differ by sex. It is known that in females, membrane receptors that are coupled to mGluR mediate the

rapid effects of estradiol in the DLS, but this mechanism has not been investigated in males [22,24]. Whether there are sex differences in the signaling pathways mediating the effects of estradiol in the DLS needs to be explored. Together, these data suggest that the sex-specific behavioral outcomes of GPER1 manipulation are likely due to differences in the downstream effects of receptor activation, rather than sex differences in overall expression.

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 DLS is sufficient to alter cocaine conditioned place preference in males. Based on the role of estradiol seen in female drug-seeking, we historically hypothesized an increase in motivated behaviors after estradiol treatment, and therefore designed studies to detect an increase in drug-seeking or drug preference, rather than a decrease. This could be one reason that a role for estradiol in drug-seeking in males has been missed until now. 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.

A

	Treatment	Male	Female
CPP	ICI (ERα/β antagonist; GPER1 agonist) (10mg/kg cocaine)	8	8
	G1 (GPER1 agonist) (10mg/kg cocaine)	8	8
	CHOL (10mg/kg cocaine)	9	10
	G15 (GPER1 antagonist) (5mg/kg cocaine)	8	--
	CHOL (5mg/kg cocaine)	9	--
SACC vs. H_2O	G1 (GPER1 agonist)	6	6
	CHOL	6	6
qPCR	*naïve*	8	8

B

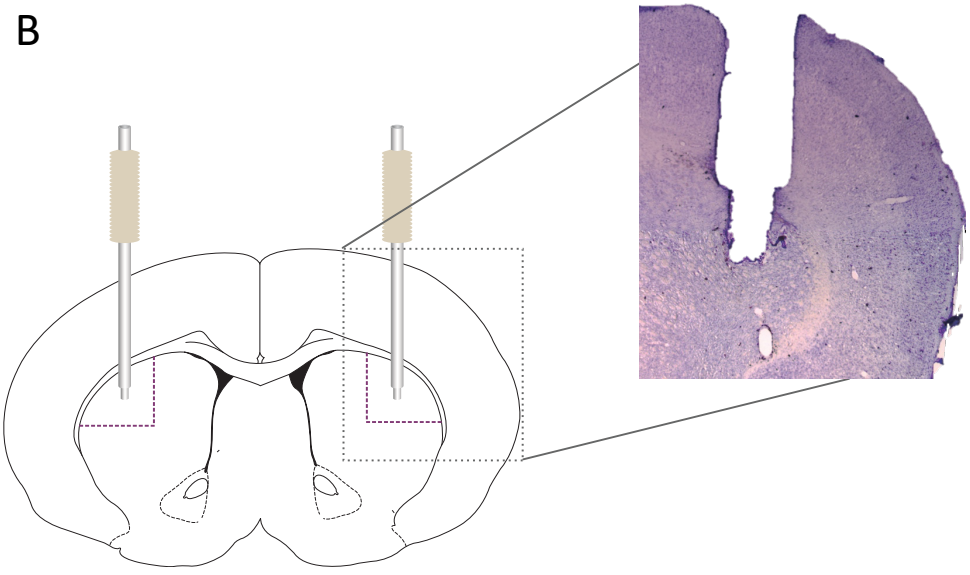


Figure 3 Pharmacological manipulation of the DLS.

(A) Number of male and female animals per treatment condition for each experiment. (B) Representative cannula placement into the DLS.

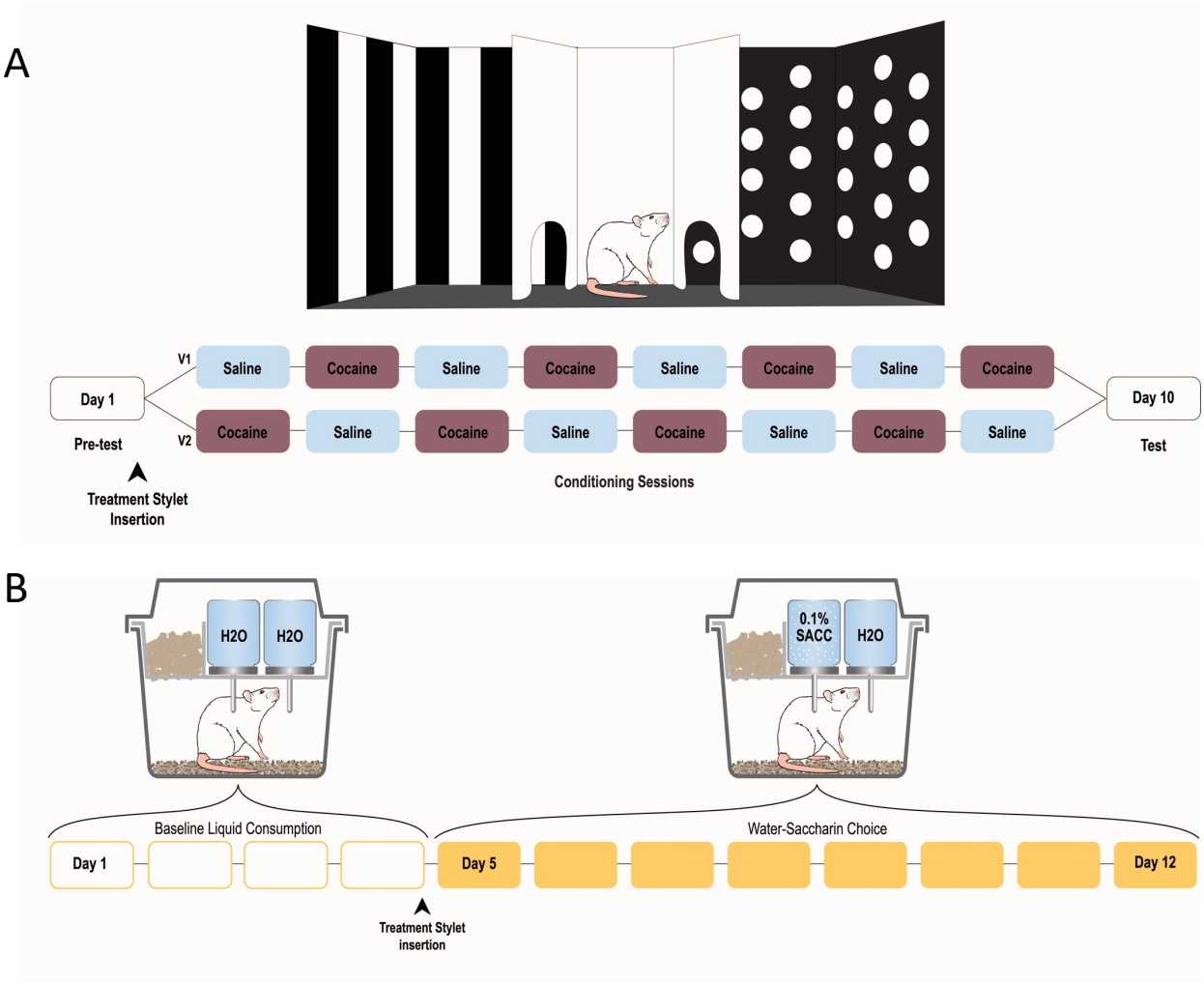

A
Day 1
Pre-test
V1
V2
Saline
Cocaine
Saline
Cocaine
Saline
Cocaine
Saline
Cocaine
Cocaine
Saline
Cocaine
Saline
Cocaine
Saline
Cocaine
Saline
Day 10
Test
Treatment Stylet Insertion
Conditioning Sessions
B
H2O
H2O
0.1% SACC
H2O
Baseline Liquid Consumption
Water-Saccharin Choice
Day 1
Day 5
Day 12
Treatment Stylet insertion

Figure 4 Behavioral paradigms to assess preference for cocaine and saccharin.

(A) The apparatus used for conditioned place preference training and testing was a three-compartment chamber with three areas, differentiated by distinct tactile and visual cues. As described in the timeline, the pre-test took place on day 1, followed by 8 conditioning sessions, ending with a final test session. Version 1 (V1) and version 2 (V2) refer to conditioning either beginning with saline or cocaine on day 2, which was counterbalanced across animals. Treatment stylets were inserted after pre-test on day 1 and remained inserted for continuous treatment through the final test session. (B) A two-bottle choice paradigm was utilized to determine preference for 0.1% SACC versus plain water. Two bottles were accessible to individually housed animals in their home cages. On days 1-4, both bottles contained H_2O only. During days 5-12, one bottle contained H_2O and the other contained 0.1% SACC dissolved in H_2O. Treatment stylets were inserted before SACC introduction on day 5 and remained for the duration of the experiment.

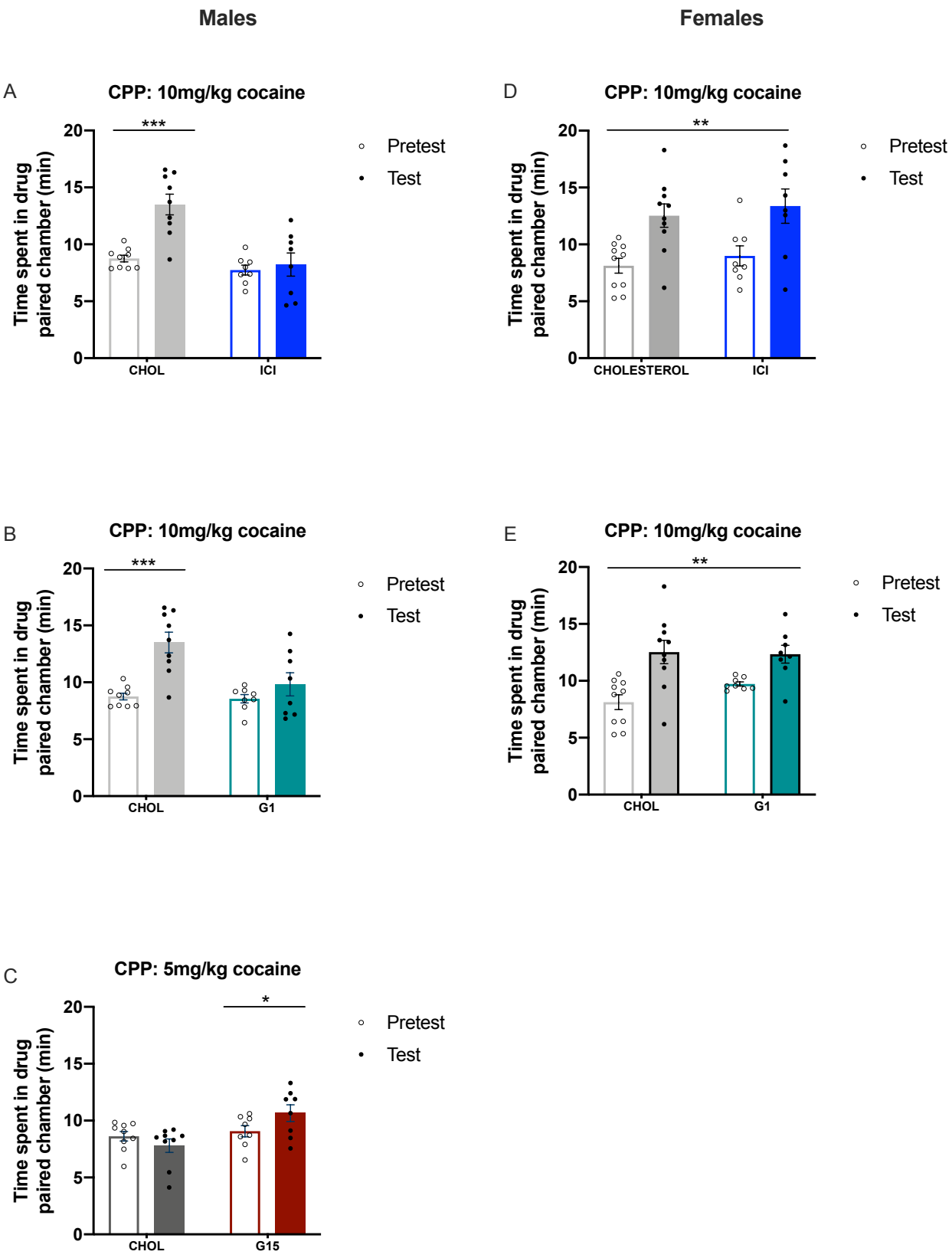

Males
Females
A
CPP: 10mg/kg cocaine
***
Time spent in drug paired chamber (min)
20
15
10
5
0
CHOL
ICI
Pretest
Test
D
CPP: 10mg/kg cocaine
**
CHOLESTEROL
ICI
Pretest
Test
B
CPP: 10mg/kg cocaine
***
CHOL
G1
Pretest
Test
E
CPP: 10mg/kg cocaine
**
CHOL
G1
Pretest
Test
C
CPP: 5mg/kg cocaine
*
CHOL
G15
Pretest
Test

Figure 5 GPER1 mediates cocaine CPP in males.

GPER1 activation/inhibition intra-DLS alters cocaine CPP for males but not females. A cocaine CPP is inferred if animals spend more time in drug-paired chamber during test versus pre-test. **(A)** At a conditioning dose of 10mg/kg cocaine, CHOL (control) treated males form a CPP however, treatment of ICI (nonselective ERα/ERβ antagonist; GPER1 agonist) or **(B)** G1 (selective GPER1 agonist) attenuates males CPP for cocaine. **(C)** Treatment of G15 (selective GPER1 antagonist) causes a CPP for a 5mg/kg dose of cocaine; CHOL treated males do not form a CPP. **(D-E)** Females treated with CHOL, ICI, and G1 form a CPP for 10mg/kg cocaine. CPP data are shown as mean +/- SEM and each point corresponds to an individual animal. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

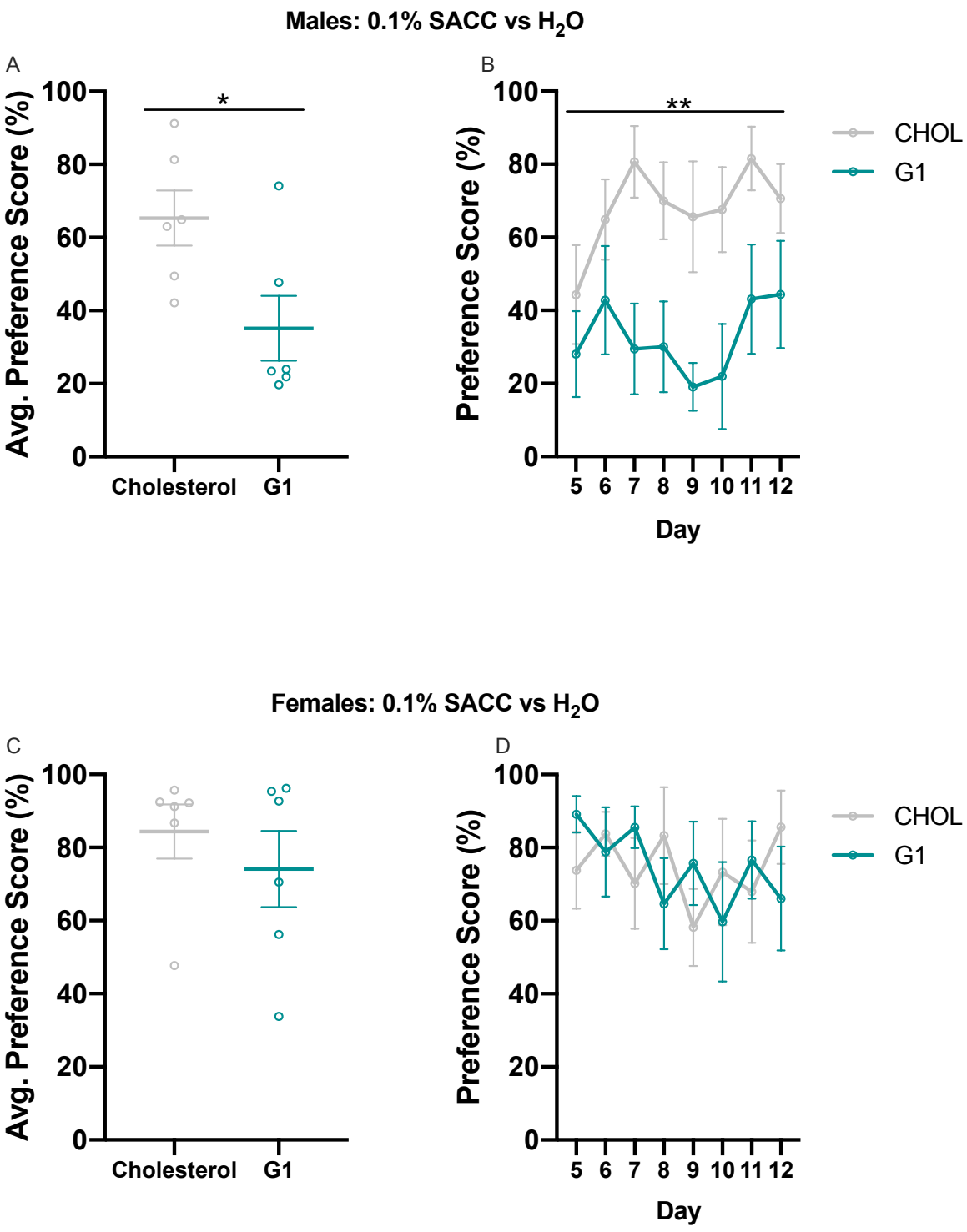
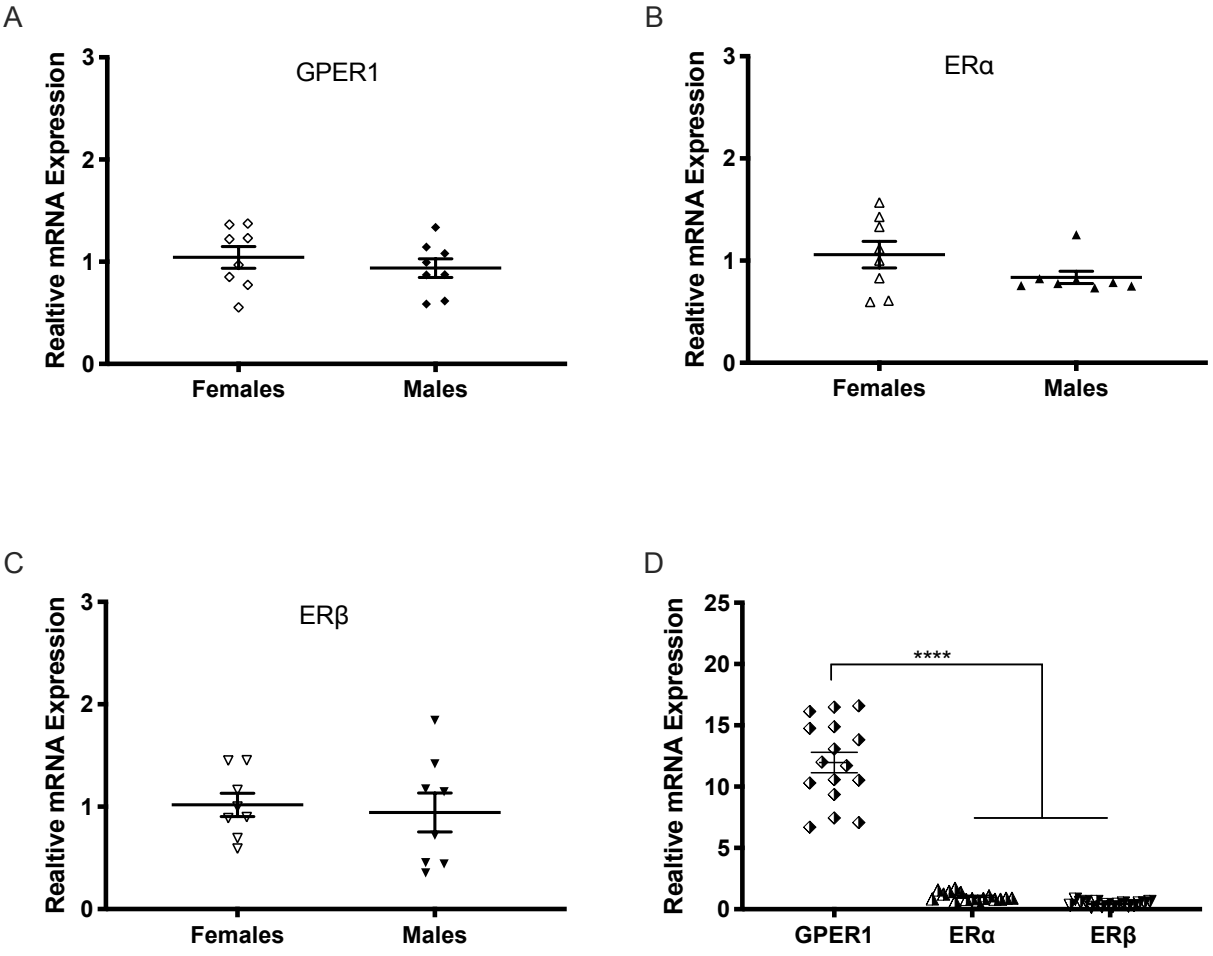

Males: 0.1% SACC vs H2O
A
B
Avg. Preference Score (%)
100
80
60
40
20
0
*
Cholesterol
G1
Preference Score (%)
**
5 6 7 8 9 10 11 12
Day
CHOL
G1
Females: 0.1% SACC vs H2O
C
D
Avg. Preference Score (%)
Cholesterol
G1
Preference Score (%)
5 6 7 8 9 10 11 12
Day
CHOL
G1

Figure 6 GPER1 mediates saccharin preference in males.

GPER1 activation intra-DLS attenuates preference for 0.1% SACC versus H_2O for males but not females. (A) G1 treatment causes a conditioned avoidance of 0.1% SACC, as indicated by a preference score < 50%. G1 treated males have a significantly lower preference score, averaged across days, than CHOL (control) males. (B) Both G1 and CHOL (control) treated females similarly formed a preference for 0.1% SACC over H_2O, as indicated by group preference scores exceeding 50%.
Preference score data are shown as mean +/- SEM with data points representing individual days. *$p < 0.05$, **$p < 0.01$.


A
GPER1
Realtive mRNA Expression
3
2
1
0
Females
Males
B
ERα
Realtive mRNA Expression
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2
1
0
Females
Males
C
ERβ
Realtive mRNA Expression
3
2
1
0
Females
Males
D
Realtive mRNA Expression
25
20
15
10
5
0
****
GPER1
ERα
ERβ

Figure 7 ERα, ERβ, and GPER1 mRNA expression in the dorsal striatum.

(A-C) There are no significant differences in relative mRNA expression of estradiol receptors in the dorsal striatum between females and males. Data are shown as males relative to females. (D) Relative mRNA expression of GPER1 is greater than ERα and ERβ, which do not differ from one another. Data are shown as GPER1 and ERβ relative to ERα. Data are shown as mean +/- SEM with data points representing individual animals. ****$p < 0.0001$

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Chapter III
Activation of G-protein Coupled Estradiol Receptor 1 in the Dorsolateral Striatum Enhances Motivation for Cocaine and Drug-Induced Reinstatement in Female but Not Male Rats

Abstract

Estradiol potentiates drug-taking behaviors, including motivation to self-administer cocaine and reinstatement of drug-seeking after extinction in females, but not males. The dorsolateral stratum (DLS) is a region of the brain implicated in mediating drug-seeking behaviors and more specifically, is a target brain area to study how estradiol regulates these behaviors. The estradiol receptors α, β, and G-protein coupled estradiol receptor 1 (GPER1) are all present in the DLS. In this study the effects of activating GPER1 in the DLS on drug-seeking are investigated.

Gonad-intact male and female rats were trained to self-administer cocaine (0.4 mg/kg/inf) on a fixed-ratio 1 schedule of reinforcement. For four weeks, animals underwent testing on a progressive ratio schedule of reinforcement to determine their motivation to attain cocaine. Halfway through progressive ratio testing, a selective agonist targeting GPER1 (G1) was administered intra-DLS to determine the contribution of GPER1 activation on motivation for cocaine. The effects of intra-DLS GPER1 activation on drug-induced reinstatement after extinction was subsequently determined.

Activation of GPER1, via G1 administration intra-DLS potentiated females' motivation to self-administer cocaine. There was no effect of prior G1 treatment on extinction of cocaine-taking in females, however, G1 treatment resulted in greater drug-

induced reinstatement (10 mg/kg cocaine, i.p.). There were no effects of intra-DLS GPER1 activation observed on motivation for cocaine or cocaine-induced reinstatement of responding in males.

These results support the conclusion that activation of GPER1 in the DLS enhances cocaine seeking behaviors for female, but not male rats.

Introduction

The prevalence of adults who will develop a substance use disorder (SUD) is approximately 10%, although a much greater percentage of individuals will have exposure to elicit drug use at some point in their lifetime (Grant et al., 2016). Many factors contribute to individual differences in escalation of drug taking behavior and the propensity towards addiction. Biological sex is one component that affects individual differences in vulnerability to develop a SUD to psychostimulants, in particular (Quigley et al., 2021). For example, women escalate cocaine use more rapidly, report greater craving for cocaine, and have shorter cocaine-free periods compared to men (Elman, Karlsgodt, & Gastfriend, 2001; Westermeyer, Kopka, & Nugent, 1997). Women also have greater incidence of relapse, possibly due to stress-induced drug seeking that occurs more in women than men (Back, Brady, Jackson, Salstrom, & Zinzow, 2005; McKay, Rutherford, Cacciola, Kabasakalian-McKay, & Alterman, 1996).

There are sex differences in rodent models of addiction that are comparable to what is reported in the clinical literature (Becker & Koob, 2016). Female rats acquire cocaine self-administration more rapidly than males do, are more motivated to obtain cocaine, and take longer to extinguish cocaine-seeking behavior, compared to males (Kippin et al., 2005; W J Lynch & Carroll, 1999; Wendy J Lynch, 2008; Roth & Carroll, 2004). In females, but not males, the presence of estradiol potentiates sensitization to cocaine, acquisition and maintenance levels of drug intake, and reinstatement of cocaine-taking after extinction (Doncheck et al., 2018; Jackson, Robinson, & Becker, 2006; Martinez et al., 2016; Zhao & Becker, 2010). Together, these data support that

estradiol plays a role in increasing vulnerability to addiction-like behaviors in female rodents.

Recent evidence supports a modulatory role of estradiol on males' preference for cocaine. Specifically, activation of the estradiol receptor subtype, G-protein coupled estradiol receptor 1 (GPER1), decreases conditioned place preference for cocaine and morphine in male rodents (Quigley & Becker, 2021; Sun et al., 2020). As mentioned above, no studies thus far have determined an effect of estradiol treatment on males' self-administration of cocaine, but this could be because prior studies have not investigated the contribution of individual estradiol receptor subtypes to drug self-administration in either sex.

Estradiol receptor subtypes including ERα, ERβ, and GPER1 are all expressed in the dorsal striatum of both males and females (Almey, Milner, & Brake, 2016; Krentzel, Willett, Johnson, & Meitzen, 2021; Quigley & Becker, 2021). Given the recent evidence implicating GPER1 as an important neuronal target for mediating the rewarding properties of cocaine in males, this study was designed to determine whether GPER1 activation within the dorsolateral striatum (DLS) modulates motivation for cocaine in either sex. The current study used a progressive ratio self-administration paradigm to determine the contribution of GPER1 activation on motivation for cocaine, and also evaluated the impact of DLS-GPER1 activation on drug-induced reinstatement in both female and male rats.

Materials and Methods

Animals

A total of 25 male and 26 female gonad-intact Sprague-Dawley rats were used in this experiment. Animals were ordered from Charles River Breeding Laboratory (Portage, MI, USA) and were approximately 75 days old on arrival. Animals were maintained on a 14:10 light/dark cycle in a temperature-controlled climate of 72°F ± 2°F. Animals were housed individually in standard ventilated cages in the laboratory vivarium. In their home cages, 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). All animals were weighed daily to determine good health and females were also vaginally lavaged daily to track and ensure that estrous cycle remained consistent. 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 Use and Care of Animals Committee.

Stereotaxic Surgery and Treatment Stylets

One week after arriving in the laboratory, rats underwent surgery for the implantation of bi-lateral guide cannula (purchased from P1 Technologies) aimed at the DLS (AP: +0.4 ML: +/-3.6 DV: -4.0). During surgery, 33-gauge solid stylets were inserted into the 26-gauge hollow guide cannula that were fixed on the animal's heads. These stylets were flush with the bottom of the guide cannula and did not protrude further into the brain. Hollow treatment stylets, however, protruded from the guide cannula by 1mm and delivered treatment directly into the DLS. Control animals received 100% cholesterol (CHOL) and experimental animals received the selective GPER1 agonist G1, in cholesterol (10% G1:90% CHOL). In order to insert stylets, rats were

briefly anesthetized with 5% isoflurane. Post-mortem analyses confirmed correct placement of guide cannula into the DLS; no animals' were excluded from analyses due to incorrect placement.

On the day of surgery, rats were injected with carprofen (5 mg/kg s.c.) and 30 minutes later were anesthetized with ketamine (50 mg/kg i.p.) and dexmedetomidine (0.25 mg/kg i.p.), then prepared in a stereotaxic frame. At the conclusion of the surgery, animals received atipamezole hydrochloride (0.5 mg/kg i.p.) and 3 ml 0.9% saline (s.c.). Every 24 hours for three days post-surgery, animals were given carprofen (5 mg/kg s.c.) prophylactically for post-operative pain then monitored for an additional seven days.

Stylets were prepared as previously described (Becker, Snyder, Miller, Westgate, & Jenuwine, 1987). Pharmacological drugs were obtained from the following sources: Cholesterol (Santa Cruz Biotechnology, purity ≥ 92%); G1 (Cayman Chemical, purity ≥ 98%). Previous studies report that G1 has no binding affinity for ERα or ERβ (Albanito et al., 2007; Bologa et al., 2006).

Catheter Surgery

One week after undergoing stereotaxic surgery, animals were fitted with indwelling jugular catheters that connected to a dorsal external port (Cummings et al., 2011). On the day of surgery, animals received carprofen (5 mg/kg s.c.) and 30 minutes later were anesthetized with 5% isoflurane in oxygen. Every 24 hours for three days post-surgery, animals were given carprofen (5 mg/kg s.c.) prophylactically for post-operative pain. Animals were monitored for an additional seven days before beginning self-administration behavioral testing.

Beginning two days after surgery and continuing everyday thereafter, catheters were flushed with 0.2 ml of gentamicin (3 mg/ml) and heparin (20 U/ml) to prevent infection and clotting, respectively. Prior to the beginning of each cocaine self-administration session, the catheters were also flushed with 0.1 ml of sterile saline. Once weekly, catheter patency was verified using 2.5 mg/kg methohexital sodium in sterile saline. Approximately 10% of animals were removed from the experiment due to catheter failure.

Cocaine Self-Administration Procedures Chamber

Cocaine self-administration was performed in standard operant chambers (Med Associates, Inc., Georgia, VT, USA) for a maximum of four hours per day, five days per week. As depicted in Figure 8, each rat was able to move freely in the operant chamber, while connected to an infusion syringe via their dorsal catheter port. A house light turned on inside the chamber to signify the start of each self-administration session. Each chamber was also equipped with two nose poke ports. The active port had an illuminated light, while the other port had no light and was therefore “inactive”. A nose poke response in the active port resulted in an intravenous 50-µl infusion of 0.4 mg/kg/infusion cocaine HCl delivered over 2.8 seconds. There was no consequence of poking in the inactive port.

Training

Animals were tested 5 days a week with 2 days off each week . During week one, rats were trained to nose poke in the active port to self-administer cocaine on a fixed-ratio 1 schedule of reinforcement. Under this schedule, a response into the active port resulted in one infusion of cocaine followed by a 5-second timeout period of drug

unavailability. If an animal nose poked during a timeout period, the nose poke was recorded but the animal did not receive an infusion of cocaine. Each training session was 3 hours long or until an animal received a maximum of 15 infusions of cocaine. If an animal did not meet the 15-infusion threshold, they were given the remaining infusions one minute apart. By day 5 of training, all animals were earning 15 infusions of cocaine.

Progressive Ratio

For four consecutive weeks thereafter, animals underwent a progressive ratio schedule of reinforcement that escalated through an exponential series: 1, 3, 6, 9, 12, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, … (Richardson & Roberts, 1996). On this schedule, the number of nose pokes required increased exponentially and the consequence remained at a single cocaine infusion (0.4mg/kg/infusion). The final completed response ratio represents the animals breaking point. All progressive ratio tests lasted 4 hours or until 1 hour elapsed without the animal having earned the next infusion.

During weeks 3 and 4 of progressive ratio self-administration, animals received either G1 or CHOL intra-DLS (see Table 2 for treatment condition assignments) via their treatment stylets. Treatment conditions were assigned so that the average breaking point between each group did not differ for weeks 1 and 2 of progressive ratio testing. Treatment stylets were inserted after the final self-administration session of week 2 and remained through week 4, except for when they were briefly replaced with new stylets between weeks 3 and 4, in order to maintain a stable dose. Treatment stylets were removed at the conclusion of the last session of week 4.

Extinction and Reinstatement

During week 5, rats underwent 1-hour extinction training twice per day for a total of 10 extinction training sessions in five days. Chamber conditions (i.e., house light and nose port light) were the same as during progressive ratio testing, however rats did not receive an infusion of cocaine after nose poking. The rate of extinction was calculated as the difference between activate and inactive nose pokes per session.

New treatment stylets were introduced after the final extinction session. Treatment assignments were counterbalanced with prior G1 or CHOL exposure, to control for confounding effects of prior pharmacological manipulation. On day one of week 6, animals were tested for drug-induced reinstatement. At the start of the self-administration session, each animal received a 10 mg/kg i.p. injection of cocaine. Similar to during extinction, number of nose pokes were recorded, however no consequence resulted from nose poking in either port.

Statistics

All statistical analyses were performed using GraphPad Prism v8.0 and IBM SPSS Statistics v27.0. Data were analyzed for general normality using the Shapiro-Wilk test but no corrections were needed. Muuchly's Test was used to determine sphericity and a Greenhouse-Geisser correction used where sphericity was violated. Effect sizes for these tests are reported as Cohen's d (d) and partial eta squared (n^2p). The threshold for significance for all statistical tests was set to $p<0.05$.

Sex differences in motivation were assessed across time, using a two-way repeated measures ANOVA (sex x session) and as average group differences, by using an unpaired t-test (Figure 9 A-B). Two-way repeated measures ANOVAs were also

used to assess the effects of G1 versus CHOL on motivation within each sex (Figure 10 A-B). Three-way repeated measures ANOVAs were used to analyze sex differences in the effects of G1 versus CHOL on motivation (Figure 11) and extinction (Figure 12). A two-way ANOVA was used to analyze sex differences in the effects of G1 or CHOL on reinstatement (Figure 13). In the case of a significant interaction, a Bonferroni multiple comparison test determined if there were significant group differences.

Finally, the effects of estrous cycle on motivation were analyzed by grouping non-estrous (metestrus and diestrus) versus estrus (proestrus and estrous) and comparing them using paired non-parametric Wilcoxon tests for week 1 and week 2 (Figure 14).

Results

During weeks 1 and 2 of progressive ratio testing, prior to any pharmacological manipulation, motivation for cocaine increases for both sexes (Figure 9 A). A two-way repeated measures ANOVA found a main effect of test session ($F_{(1.456,72.79)} = 8.197$; $p = 0.0020$; $n^2p = 0.141$). An unpaired t-test was performed to compare the average breaking point values for males versus females across weeks 1 and 2 ($t_{(18)} = 2.412$; $p = 0.0267$; $d = 1.078$) (Figure 9 B). This outcome suggests that the average breaking point for females is greater than males in the first two weeks of self-administration.

During weeks 3 and 4 of progressive ratio testing, DLS-GPER1 receptors were pharmacologically activated using G1 and motivation for cocaine was assessed within each sex (Figure 10 A-B). A two-way repeated measures ANOVA revealed a main effect of treatment for females ($F_{(1,24)} = 4.267$; $p = 0.0498$; $n^2p = 0.1509$) but no main

effect of day, and no treatment x day interaction. For males, there was no main effect of treatment or day, and no significant interaction.

As illustrated in Figure 11, there are sex differences in the effects of G1 on breaking point for cocaine. A three-way repeated measures ANOVA revealed main effects of both sex ($F_{(1,47)} = 6.973$; $p = 0.0112$; $n^2p = 0.129$) and timepoint ($F_{(1,47)} = 33.14$; $p < 0.0001$; $n^2p = 0.414$). Additionally, there was a significant 3-way interaction among sex x treatment condition x timepoint ($F_{(1,47)} = 5.654$; $p = 0.0215$; $n^2p = 0.107$). Bonferroni multiple comparisons discovered significant group differences between G1 treated males and females' post-treatment ($p = 0.0039$) as well as a significant difference in breaking point between timepoints in females treated with G1 ($p < 0.0001$).

As illustrated in Figure 12, there was no effect of prior G1 exposure on rates of extinction. A three-way repeated measures ANOVA revealed a main effect of day ($F_{(9,243)} = 5.840$; $p < 0.0001$; $n^2p = 0.178$) and a main effect of treatment condition ($F_{(1,27)} = 4.317$; $p = 0.0474$; $n^2p = 0.138$). There were two significant interactions: sex x day ($F_{(9,243)} = 2.563$; $p = 0.0078$; $n^2p = 0.087$) and sex x treatment condition ($F_{(9,243)} = 2.982$; $p = 0.0022$; $n^2p = 0.099$). Bonferroni multiple comparisons indicated that the G1 females were significantly different from CHOL females ($p < 0.0001$) and both groups of males ($p < 0.0001$) on day 1 only. There were no group differences on any other day of extinction training between or within either sex.

Females treated with G1 also exhibited greater drug-induced reinstatement than did males (Figure 13) regardless of prior treatment. A two-way ANOVA revealed a main effects of treatment condition ($F_{(1,24)} = 5.189$; $p = 0.0319$; $n^2p = 0.165$) and sex ($F_{(1,24)} = 4.745$; $p = 0.0394$; $n^2p = 0.178$). There was a significant sex x treatment condition

interaction ($F_{(1,24)} = 4.940$; $p = 0.0359$; $n^2p = 0.171$). Bonferroni multiple comparisons showed that G1 treated females were significantly different than CHOL females ($p = 0.0460$), G1 males ($p = 0.0241$), and CHOL males ($p = 0.0259$).

For females, phase of estrous cycle (metestrus/diestrus versus proestrus/estrus) had an effect on breaking point during week 1, but not during week 2 of progressive ratio (Figure 14). For each female animal, mean breaking points during metestrus/diestrus days were compared to the mean breaking points during proestrus/estrus days. A paired t-test was used to compare group means. During week 1, breaking point during proestrus/estrus was significantly greater than during metestrus/diestrus ($t_{(23)} = 4.693$; $p < 0.0001$; $d = 0.782$). There was no difference between estrous cycle timepoints during week 2 of progressive ratio ($t_{(24)} = 0.8255$; $p = 0.172$; $d = 0.094$).

Discussion

We report here a sex difference in the effects of intra-DLS GPER1 activation on cocaine self-administration. For females, activation of GPER1 enhances females' willingness to work for cocaine (i.e., breaking point), but this effect was not observed in males. Prior GPER1 activation did not alter females' or males' rates of extinction. However, females with intra-DLS GPER1 activation also show greater cocaine-induced reinstatement of drug-seeking behavior compared to control females. The effects of GPER1 activation on reinstatement in females were also not observed in males. Together, these findings indicate that estradiol may be enhancing vulnerability to addiction in females, at least in part, by acting on GPER1.

While this is the first study to show a role of GPER1 on cocaine self-administration specifically, a growing literature supports the role of estradiol in regulating female behaviors related to addiction. For example, for female rodents, drug-associated cues acquire a higher incentive value when they are initially presented during estrus versus non-estrus (Johnson et al., 2019). While the current study did not investigate the association of cue-learning, we similarly report an effect of estrous cycle during initial stages of cocaine self-administration in females. During week 1 of progressive ratio testing, females show greater motivation to attain cocaine during proestrus/estrus compared to metestrus/diestrus. The lack of effect of estrous cycle in the succeeding weeks is likely due to the enhanced propensity to take cocaine overall.

We found that there were no differences in extinction rates between males and females or between prior treatment conditions beyond day 1 of extinction training. Prior studies have shown that estradiol is necessary for learning and extinction of cocaine-seeking in females (Twining, Tuscher, Doncheck, Frick, & Mueller, 2013). Given that animals in the current study are gonad-intact and have circulating estradiol, it is not surprising that they extinguished at similar rates. It was important in the current study that animals extinguish similarly in order to compare rates of reinstatement.

Estradiol enhances females' reinstatement of cocaine self-administration (Doncheck et al., 2018). This effect had previously been shown to be regulated by ERβ, and not ERα, but this study was done via peripheral injections and did not investigate role of GPER1 on reinstatement (Larson & Carroll, 2007). Our study supports the idea that the DLS is a target region for estradiol's effects on reinstatement in females.

Sex differences in drug-taking and cocaine reward are, in part, regulated by the interactions between estradiol and the dopamine system (Calipari et al., 2017; Kokane & Perrotti, 2020; Yoest, Quigley, & Becker, 2018). In vitro studies have shown that estradiol enhances stimulated dopamine release and amphetamine-induced dopamine release in dorsal striatal tissue from female but not male rats (Becker, 1990). In vivo studies showed that peripheral estradiol treatment in gonadectomized rats increases cocaine-induced dopamine levels in the dorsal striatum of ovariectomized females but not castrated males (Cummings, Jagannathan, Jackson, & Becker, 2014). Given the direct effect of intra-DLS GPER1 activation on cocaine-seeking in females seen in this study, we hypothesize that GPER1 could be, in part, modulating the effects of estradiol on drug-induced DA release. Future studies should investigate this mechanism in both sexes.

In the current study, we did not see a protective effect of GPER1 activation on males' motivation for cocaine, as both G1- and CHOL-treated males show increased motivation over time. However, we have previously reported that intra-DLS GPER1 activation attenuates cocaine conditioned place preference in males (Quigley & Becker 2021). Previous research that demonstrated that the DLS is necessary for stimulus-response learning in males, along with the current results, suggest that the timing of pharmacological activation of intra-DLS GPER1-, relative to initial drug exposure, is important for GPER1's effects on motivation for cocaine. (Yin, Knowlton, & Balleine, 2005, 2006). In our earlier study, GPER1 receptors in the DLS were activated or inhibited prior to the initial cocaine treatment, whereas in the current study, animals begin taking cocaine three weeks prior to administration of the GPER1 agonist.

Additional studies are needed to determine whether activating GPER1 receptors intra-DLS before rats are trained to self-administer cocaine would affect the subsequent motivation and propensity to self-administer in males and females.

As discussed above, in our prior study we reported that intra-DLS GPER1 attenuated males' preference, or "liking", of cocaine. In this study we have shown that there is no effect of intra-DLS GPER1 on "wanting" cocaine in males. The neurobiological mechanisms of "liking" a drug are discrete from "wanting"; that is, one may not necessarily like a drug but still crave and consume it. These dissociable mechanisms and are mediated by opioidergic and dopaminergic signaling, respectively (Berridge, 2007; Robinson & Berridge, 1993). We speculate that the interactions of GPER1 on opioid and dopamine signaling are different for females and males, and this could be contributing to sex dependent behavioral outcomes related to propensity to addiction.

There is circumstantial evidence for sex differences in the circuitry for "wanting" and "liking". In females, estradiol binds on GABAergic interneurons, which disinhibits dopaminergic neurons and increases dopamine levels in the striatum (Yoest, Cummings, & Becker, 2014). This enhanced neurotransmission of dopamine is presumably responsible for females' more rapid escalation of self-administration and enhanced motivation to attain psychostimulants (Cummings et al., 2014; Song, Yang, Peckham, & Becker, 2019). Directly below the dorsal striatum is the nucleus accumbens shell which is an opioid hedonic hotspot that regulates "liking" (Castro & Berridge, 2014). In males, pharmacological studies have implicated mu-opioid receptor functioning in the shell subregion to regulate responses for palatable food and cocaine

(Simmons & Self, 2009; Ward, Nicklous, Aloyo, & Simansky, 2006). The direct interactions of GPER1 on μ-opioid receptor function in the dorsal and ventral striatum are yet to be investigated. However, there is some evidence for crosstalk between these receptors including GPER1 activation rapidly downregulating μ-opioid receptors in the arcuate nucleus as well as eliciting phosphorylation of μ-opioid receptors in human neuroblastoma SH-SY5Y cells (Ding et al., 2019; Long, Serey, & Sinchak, 2014).

In summary, the present study confirmed previous findings that there are sex differences related to motivation to attain drugs of abuse. As discussed above, a large body of work has supported that estradiol enhances females' vulnerability towards addiction but has not necessarily unveiled which estradiol receptor subtypes are responsible for the behavioral effects seen in females. The results of this study support a novel role of GPER1 in females and provides a future target for preclinical research as well as clinical research targeted at therapeutics for addiction.

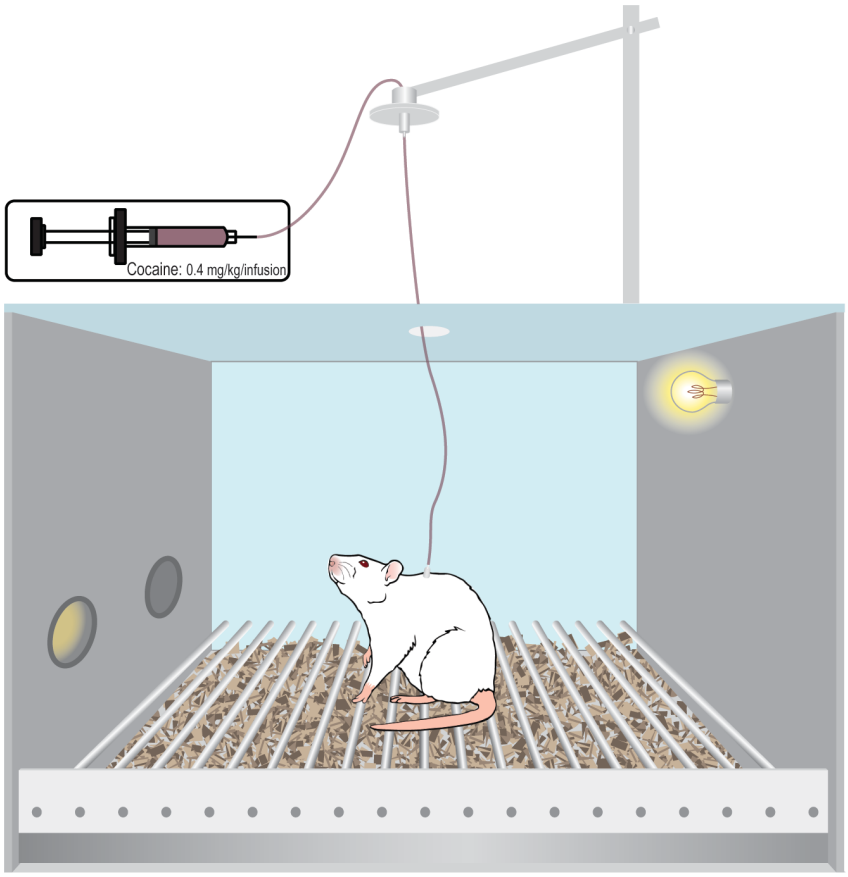
Perspectives and Significance

It is vital that we better understand the neurobiological mechanisms contributing to relapse in women, given that they are more sensitive to environmental cues and more susceptible to spontaneous relapse (Janes et al., 2010; Quigley et al., 2021). Increased drug-seeking induced by estradiol in females has been well established and the current study aids to this body of knowledge by identifying a role for GPER1 specifically. In this study, activation of GPER1 in the DLS not only enhances motivation for cocaine in females, but also increases drug-induced reinstatement. The information gained here may be used to target treatment for addiction via selective estradiol receptor modulators.

		Progressive Ratio		Extinction	Reinstatement
		Weeks 1 & 2: Pre-treatment	Weeks 3 & 4: w/ Treatment	Week 5: No Treatment	Week 6 (1 Day): w/ Treatment
Males	G1	n = 13	n = 13	(prior G1) n = 9	n = 8
	Cholesterol	n = 12	n = 12	(prior CHOL) n = 8	n = 8
Females	G1	n = 12	n = 12	(prior G1) n = 6	n = 6
	Cholesterol	n = 14	n = 13	(prior CHOL) n = 8	n = 6

Table 2 Treatment condition assignments for self-administration.

This table presents subjects per treatment condition at each stage of the self-administration paradigm.


Cocaine: 0.4 mg/kg/infusion

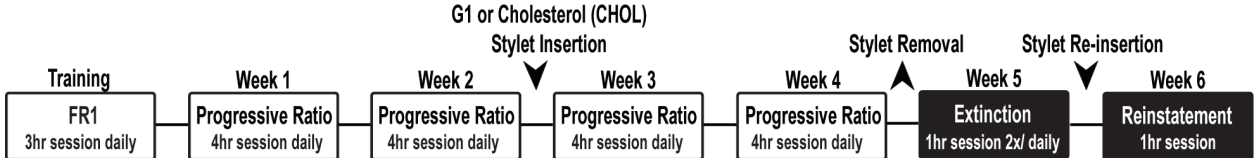
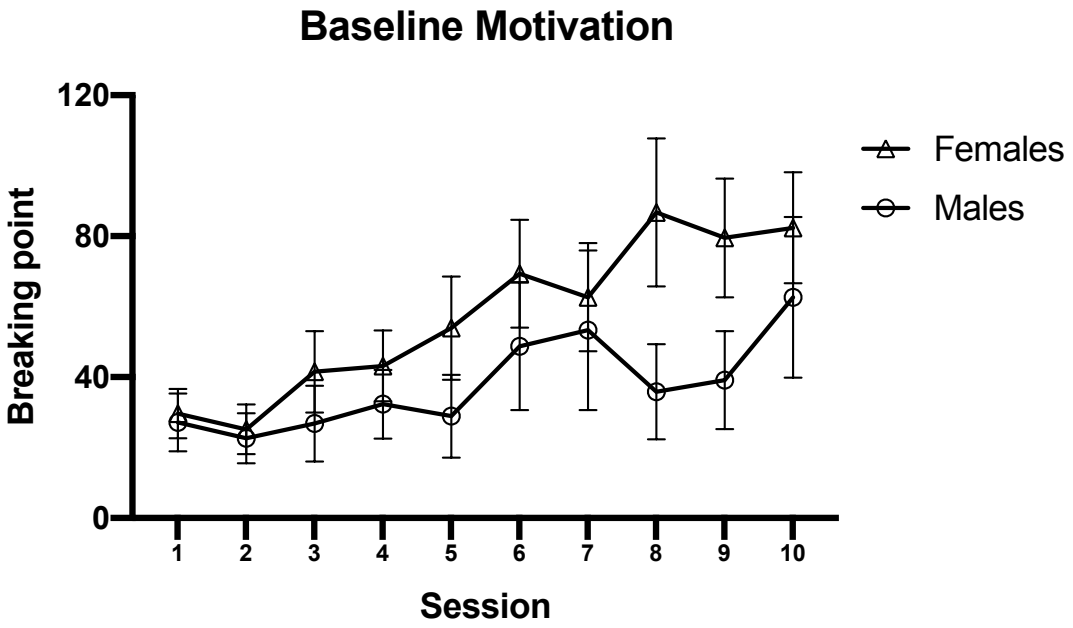

G1 or Cholesterol (CHOL)
Stylet Insertion
Stylet Removal
Stylet Re-insertion
Training
FR1
3hr session daily
Week 1
Progressive Ratio
4hr session daily
Week 2
Progressive Ratio
4hr session daily
Week 3
Progressive Ratio
4hr session daily
Week 4
Progressive Ratio
4hr session daily
Week 5
Extinction
1hr session 2x/ daily
Week 6
Reinstatement
1hr session

Figure 8 Operant conditioning chamber and behavioral paradigm.

Illustration of self-administration operant chamber and timeline for self-administration training, progressive ratio, extinction and reinstatement testing.

A


Baseline Motivation
120
80
40
0
Breaking point
1
2
3
4
5
6
7
8
9
10
Session
Females
Males

B

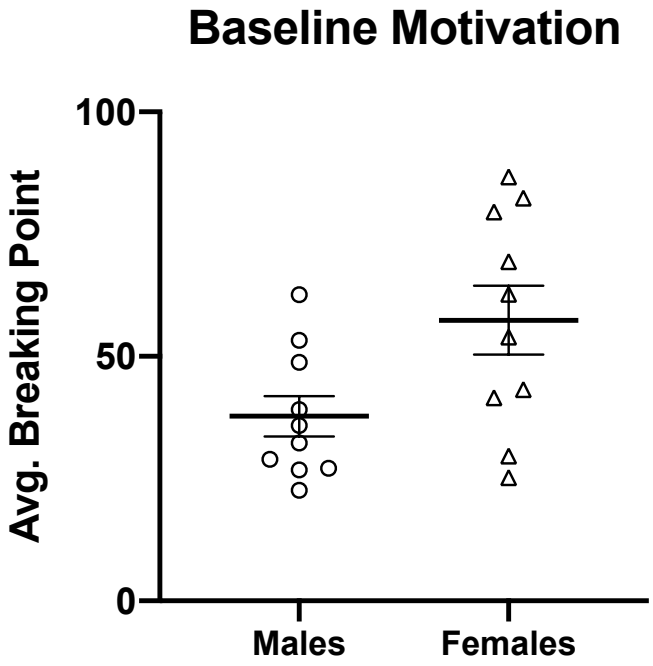
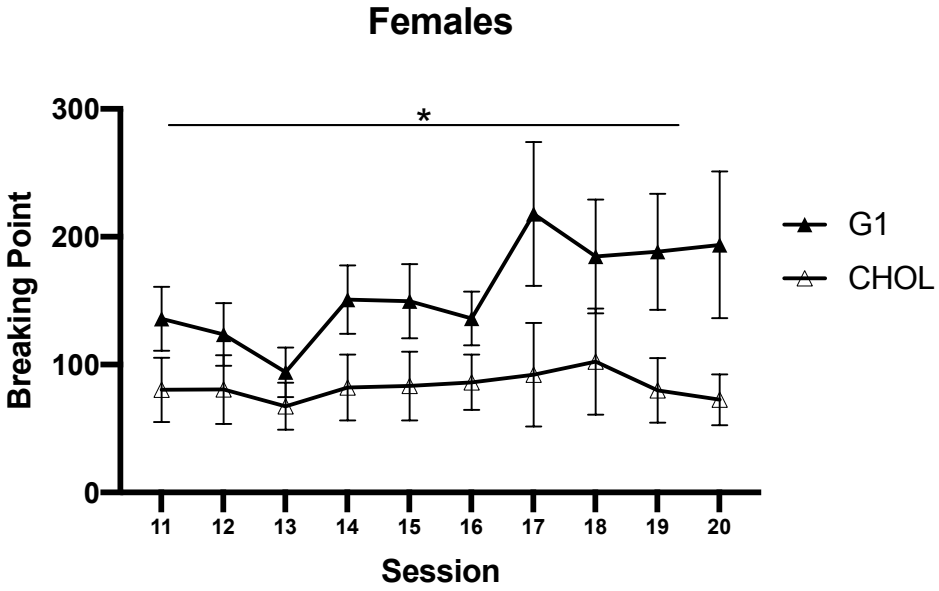

Baseline Motivation
100
50
0
Avg. Breaking Point
Males
Females

Figure 9 Baseline motivation for cocaine.

During weeks 1 and 2 of progressive ratio testing, (A) breaking point increases across self-administrations session for both sexes ($p < 0.0001$). (B) Females' have a greater average breaking point across weeks compared to males ($p = 0.0267$). Data are presented as mean ± SEM.

A


Females
*
300
200
100
0
Breaking Point
11
12
13
14
15
16
17
18
19
20
Session
G1
CHOL

B

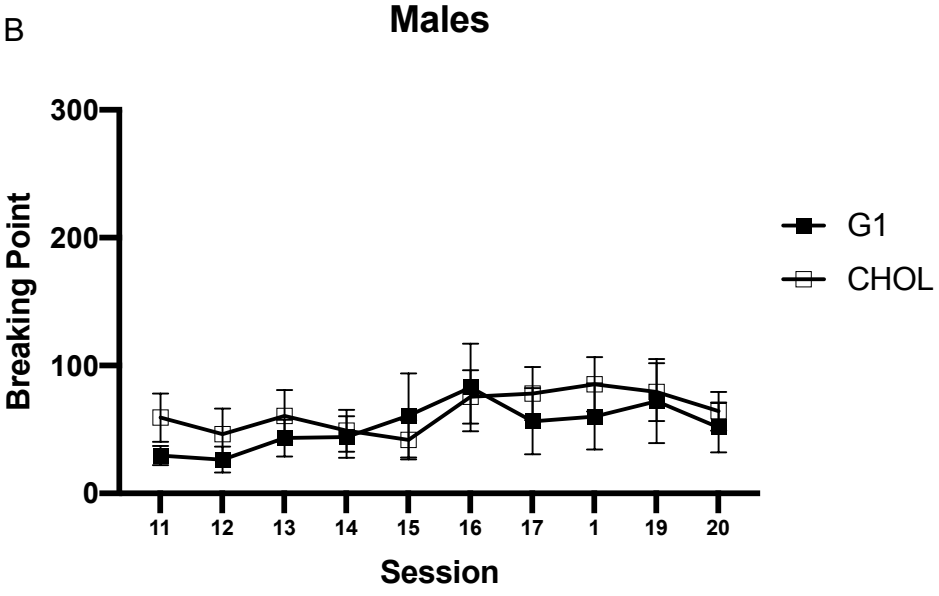

Males
300
200
100
0
Breaking Point
11
12
13
14
15
16
17
1
19
20
Session
G1
CHOL

Figure 10 Effects of DLS-GPER1 activation on motivation for cocaine in females and males.

During weeks 3 and 4 of progressive ratio testing, G1 potentiates motivation for cocaine in (A) females ($p = 0.0498$) but not (B) males. Data are presented as mean ± SEM.

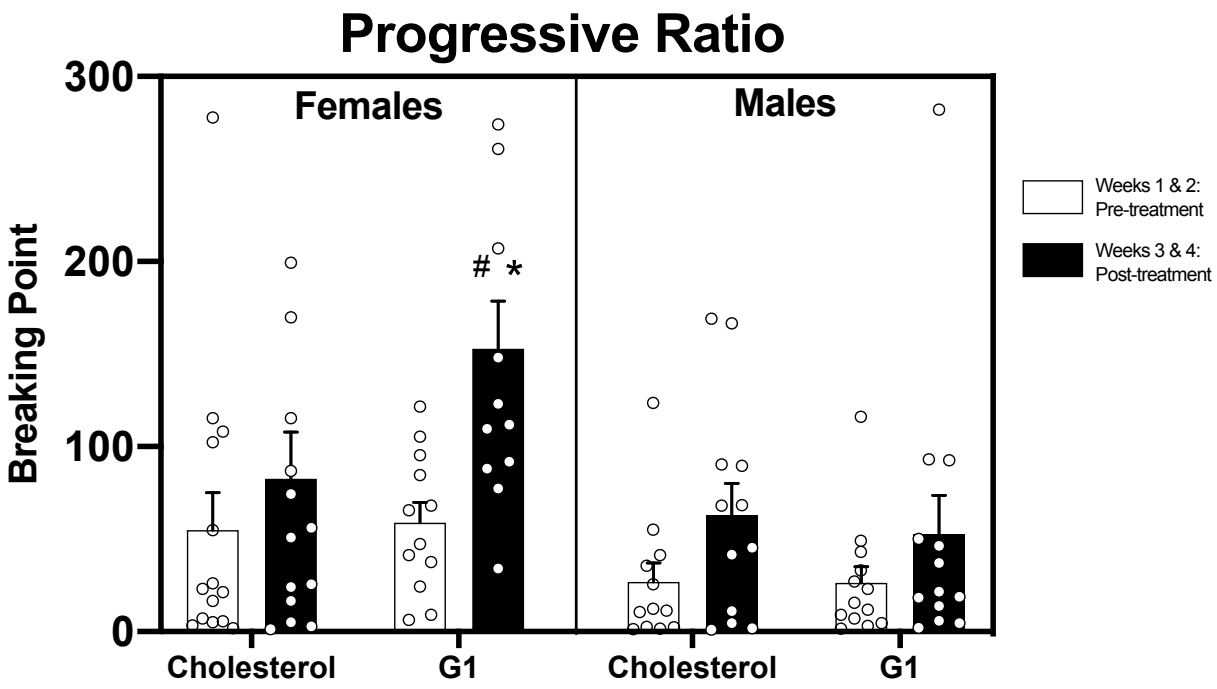

Progressive Ratio
Females
Males
Breaking Point
300
200
100
0
*
Cholesterol
G1
Cholesterol
G1
Weeks 1 & 2:
Pre-treatment
Weeks 3 & 4:
Post-treatment

Figure 11 Sex differences in the effects of DLS-GPER1 activation on motivation for cocaine.

There are sex differences in the effects of GPER1 activation on motivation for cocaine. During weeks 3 and 4 of progressive ratio (PR), G1 treated females have significantly greater breaking point (than they did during weeks 1 and 2, prior to treatment ($p < 0.0001$). G1 treated females also have a greater breaking point than G1 treated males, during weeks 3 and 4 of PR ($p = 0.0039$). Data are presented as mean ± SEM.

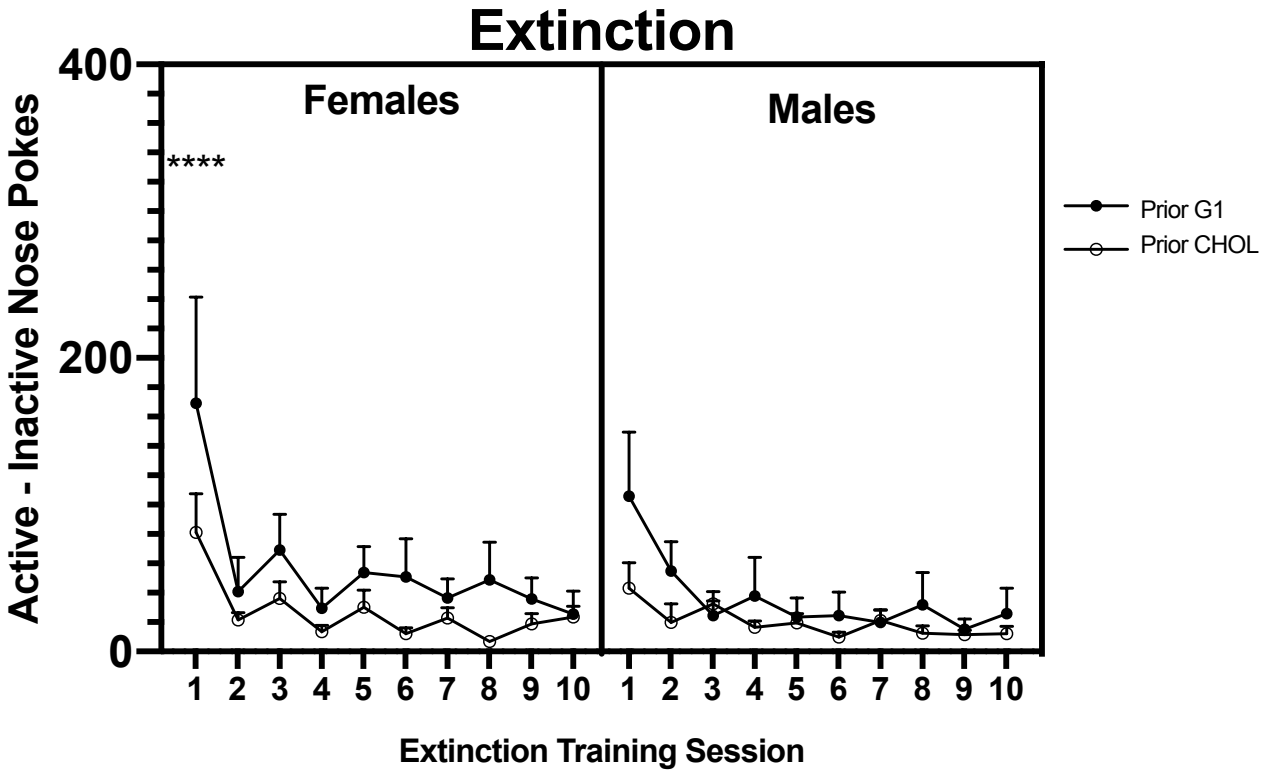

Extinction
Females
Males
****
400
200
0
Active - Inactive Nose Pokes
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
Extinction Training Session
Prior G1
Prior CHOL

Figure 12 No sex differences in rates of extinction.

There is no effect of prior G1 treatment and no sex difference in the rates of cocaine self-administration. During the first extinction session only, prior G1 treated females are greater than all other groups ($p < 0.0001$). Data are presented as mean ± SEM.

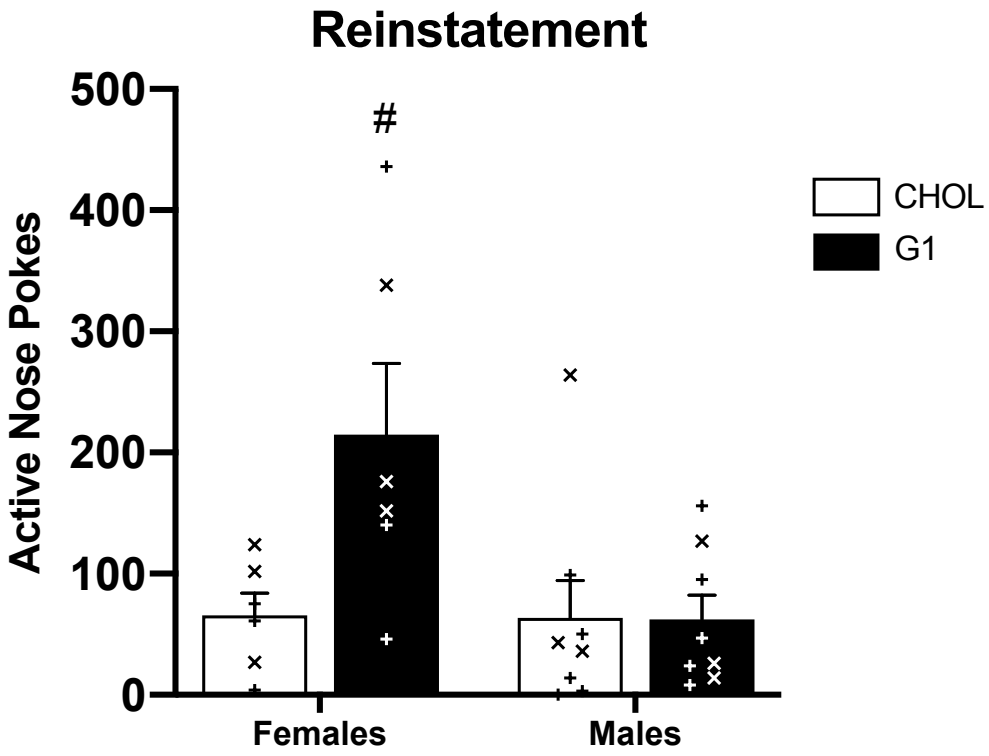
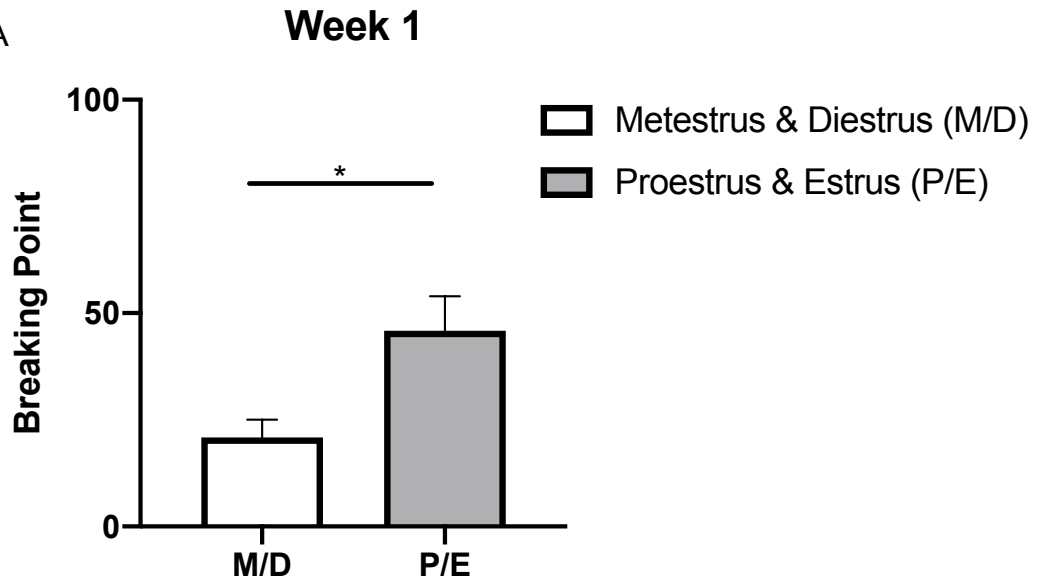

Reinstatement
Active Nose Pokes
500
400
300
200
100
0
#
Females
Males
CHOL
G1

Figure 13 Sex differences in the effects of DLS-GPER1 activation on drug-induced reinstatement of cocaine-seeking.

GPER1 activation enhances cocaine-induced reinstatement in females but not males. G1 treated females have a significantly greater number of active pokes than CHOL treated females ($p = 0.0460$), G1 males ($p = 0.0241$), and CHOL males ($p = 0.0259$). Data are presented as mean ± SEM. Individual data points presented as “X” indicate prior G1 treatment and individual data point presented as “+” indicate prior CHOL treatment, during weeks 3 and 4 of self-administration.

A


Week 1
Metestrus & Diestrus (M/D)
Proestrus & Estrus (P/E)
Breaking Point
100
50
0
*
M/D
P/E

B

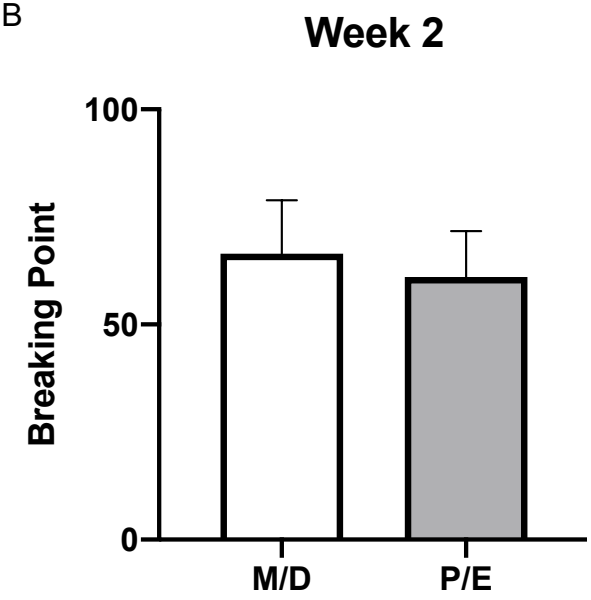

Week 2
Breaking Point
100
50
0
M/D
P/E

Figure 14 Effects of estrous cycle on motivation for cocaine.

Females' breaking point differs by phase of estrous cycle during (A) week 1 of progressive ratio self-administration ($p = 0.0001$) but not during (B) week 2. Data are presented as mean ± SEM.

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Chapter IV
Alterations of Striatal c-Fos Immunoreactivity induced by G-protein Coupled Estradiol Receptor 1 Activation

Abstract

Estradiol receptor activation has sex-specific effects on the neural mechanisms mediating reward. Prior work has suggested that, for males, activation of GPER1 in the dorsolateral striatum (DLS) decreases preference for drugs of abuse or naturally other rewarding stimuli. In this study, we investigated how intra-DLS GPER1 activation, via administration of G1, alters c-Fos immunoreactivity (IR) with and without exposure to 10 mg/kg cocaine (i.p.). Additionally, we investigated whether intra-DLS GPER1 activation alters cocaine-induced locomotor activity. We found that G1 administration attenuates cocaine-induced neuronal activation, as measured by c-Fos IR, in the dorsomedial striatum (DMS). We also found that in both the nucleus accumbens core (NAcC) and shell (NAcSh), G1 administration alone enhances c-Fos IR levels to the same degree that cocaine exposure does. We did not find an effect of either G1 treatment or cocaine exposure on c-Fos IR in the prelimbic cortex or external globus pallidus. Finally, our findings suggest G1 potentiates cocaine-induced locomotor activity. In sum, the present study provides further evidence of G1 altering cocaine-induced neural adaptations in male rats and provides site-specific regions to explore next.

Introduction

While the underlying neurocircuitry of addiction is complex, certain brain regions been identified as being important for regulating an individual's behavior and propensity towards addiction. Rodents provide an exceptional model to study the neurobiology of addiction because areas of their brain which regulate motivation and reward are similar to those of humans. One way to measure the involvement of reward circuitry is to quantify levels of c-Fos immunoreactivity (IR), as a proxy neuronal activation. C-Fos is the protein product of the c-fos immediate early that is induced by a broad range of stimuli, some of which include consumption of palatable food or drugs of abuse, exposure to novel environments, or learning and memory consolidation.

Initial exposure to psychostimulants, or other rewarding-stimuli, results in feelings of pleasure or "liking". These reactions are mediated by opioid hedonic hotspots within the limbic system, including areas within the nucleus accumbens (NAc). In particular, the shell subregion of the NAc has been shown to mediate "liking" and "disgust" reactions to sucrose as well as conditioned place preference (Castro & Berridge, 2014). Craving and continuous drug-seeking, however, occurs after the initial pleasurable effects of a drug have subsided (Robinson & Berridge, 1993). These effects are mediated by dopamine and induce "wanting", rather than opioid mediated processes involved in "liking" (Berridge, 2007, 2009; Robinson & Berridge, 1993).

One of the major dopamine pathways of the brain is the nigrostriatal circuit, which influences cognition and reward, as well as regulates purposeful movement via dopaminergic- modulation of the basal ganglia motor loops. Among facilitating motor control, motor learning, and emotional regulation, the basal ganglia is implicated in

regulating reward and drug-seeking, via the “direct” and “indirect” pathways. Briefly, the direct pathway is the projection of GABAergic medium spiny neurons (MSNs) from the dorsal striatum to the substantia nigra and the indirect pathway is the projection from the dorsal striatum to the subthalamic nucleus, via the globus pallidus (external), followed by glutamatergic transmission to the substantia nigra (Yager, Garcia, Wunsch, & Ferguson, 2015). Stimulation of direct pathway neurons ultimately dis-inhibits the thalamus and promotes behavior while stimulation of the indirect pathway suppresses thalamic activity and suppresses behavior. These direct/indirect pathways should work in unison in a “go” and “stop” fashion to balance behavior and decision making however, drugs of abuse cause adaptations within this system (Macpherson, Morita, & Hikida, 2014). Such changes include alteration of gene expression of D1 and D2 receptors in the direct and indirect pathway, respectively, which ultimately results in dysfunction and habitual behaviors, such as compulsive drug-seeking (Calabresi, Picconi, Tozzi, Ghiglieri, & Di Filippo, 2014; Heiman et al., 2008; Lobo & Nestler, 2011; Lüscher & Malenka, 2011; Wright & Dong, 2017).

There are direct effects of the gonadal hormone, estradiol, that enhance female’s behavioral responses and vulnerability towards addiction. For example, estradiol treatment enhances behavioral sensitization to cocaine in gonadectomized female, but not male rats (Hu & Becker, 2003). Estradiol treatment also causes rapid escalation of cocaine self-administration and greater motivation to attain cocaine in females (Hu & Becker, 2008; Lynch, 2008; Peris, Decambre, Coleman-Hardee, & Simpkins, 1991). Additionally, estradiol potentiates female’s reinstatement of cocaine-seeking after

extinction training, an effected which is mediated by the prefrontal prelimbic cortex (Doncheck et al., 2018; Kippin et al., 2005).

Experiments which investigated the effects of estradiol on drug-seeking, described above, did not observe effects of estradiol on males' propensity towards addiction-like behaviors. Recently, however, manipulation of one estradiol receptor subtype, GPER1, was found to have a behavioral effect in males, but not females. Activation of GPER1 in the dorsolateral striatum (DLS) decreased males' conditioned place preference for cocaine while inhibition of the receptor enhanced preference (Quigley & Becker, 2021). Another recent study concluded that GPER1 knockout facilitates morphine conditioned place preference in males (Sun et al., 2020). Not only do the effects of GPER1 appear to be mediating the rewarding properties of drugs of abuse, but GPER1 activation intra-DLS is also reported to decrease preference for saccharin, suggesting that this mechanism is not confined to drug-related stimuli (Quigley & Becker, 2021).

It is unclear how estradiol is facilitating drug-seeking for females but having an opposite effect in males. Multiple studies have shown that there are not sex differences in estradiol receptor expression in the dorsal striatum of adult rats (Krentzel, Willett, Johnson, & Meitzen, 2020; Quigley & Becker, 2021). In females, GPER1 receptors are localized to GABAergic and cholinergic neurons in the dorsal striatum, but no studies have investigated their cellular location in the male brain (Almey, Milner, & Brake, 2016; Hammond, Nelson, & Gibbs, 2011).

To gain insight on how GPER1 activation in intra-DLS could be mitigating the rewarding properties of cocaine for males, we investigated cocaine-induced neuronal

activation in reward-regions of the brain and how these patterns of activation are altered by pharmacological manipulation of GPER1. To this end, we used c-Fos IR as an indirect measure of neuronal activation in the nucelus accumbens core (NAcC) and shell (NAcSh), dorsomedial striatum (DMS), prelimbic cortex (PRL), and external globus pallidus (GPe), and. We also determined the effects of intra-DLS GPER1 activation on cocaine-induced locomotor, as a measure of an acute behavioral response to cocaine in these animals.

Methods

Animals

24 male Sprague-Dawley rats were obtained from Charles River Breeding Laboratory (Portage, MI, USA) and were approximately 75 days old on arrival. Animals were maintained on a 14:10 light/dark cycle in a temperature-controlled climate of 72°F ± 2°F, in ventilated laboratory cages. 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). Animals housed in pairs until undergoing surgery, after which they were housed individually. 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 Use and Care of Animals Committee.

Stereotaxic Surgery

One week after arriving in the laboratory, rats underwent surgery for the implantation of bi-lateral guide cannulae aimed at the DLS (AP: +0.4 ML: +/-3.6 DV: -4.0). On the day of surgery, rats were injected with carprofen (5 mg/kg s.c.) and 30

minutes later were anesthetized with ketamine (50 mg/kg i.p.) and dexmedetomidine (0.25 mg/kg i.p.), then prepared in a stereotaxic frame. During surgery, 33-gauge solid stylets were inserted into the 26-gauge hollow guide cannula that were fixed on animals' skull. These stylets were flush with the bottom of the guide cannula and did not protrude into the brain. At the conclusion of the surgery, rats were given atipamezole hydrochloride (0.5 mg/kg i.p.) and 3ml 0.9% saline (s.c.). Every 24 hours for three days post-surgery, rats were given carprofen (5 mg/kg s.c.) prophylactically for post-operative pain.

Drug Preparation

Treatment conditions were randomly assigned to animals prior to behavioral testing. Control animals received 100% cholesterol and experimental animals received 10% G1 (agonist targeting GPER1) dissolved in cholesterol, via stylets which protruded from the guide cannula by 1 mm and delivered treatment directly into the DLS. Treatment stylets were prepared as previously described (Becker, Snyder, Miller, Westgate, & Jenuwine, 1987). In order to insert stylets, rats were briefly anesthetized with 5% isoflurane.

Hollow guides and interlocking treatment stylets were manufactured by and purchase from P1 Technologies (Roanoke VA). Drugs were obtained from the following sources: G1 (Cayman Chemical (Ann Arbor, MI), purity ≥ 98 and Cholesterol (Santa Cruz Biotechnology (Dallas, TX) purity ≥ 92%).

Behavioral Testing

Animals' locomotor behavior was tested over two consecutive days. On day 1, animals were placed in a novel context (15.5 in x 12 in chamber) for 30 minutes and

their total distance traveled was recorded. Immediately after, treatment stylets (cholesterol or G1) were inserted, and animals were returned to their home cages. On day 2, animals were placed in the same context however this time, animals received an i.p. injection of 10 mg/kg cocaine or saline prior to being placed in the chamber for 30 minutes. Animals were returned to their home cage for roughly 60 minutes afterwards. ANYMAZE tracking software (Stoelting Co., Wood Dale, IL) was utilized to track distance traveled by each animal.

c-Fos Immunohistochemical Analysis

Exactly 60 minutes after receiving the injection of cocaine or saline, each animal received an injection of 0.5 ml of Sodium Pentobarbital (i.p). Once the animal was fully sedated, it received a transcardial perfusion of 0.1M phosphate buffered saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 48 hours, then transferred into 30% sucrose. Brains were sliced on a microtome in 40-micron sections, which were collected and store in cell culture wells in cryoprotectant solution at -20°C.

Immunohistochemistry protocols, as described by (Tronson et al., 2009), were used. Briefly, sections were incubated in anti-c-Fos antibody (ABCAM; ab208942; 1:500), Goat Anti-Mouse IgG antibody (Vector Laboratories; BA9200; 1:200), and DAB Chromogen (Sigma-Aldrich; D4293).

One hemisphere from each animal was imaged quantified at 10x magnification (Figure 17). For each region, three (100mm x 100mm x 40ug) boxes were placed on the coronal brain image using Adobe Photoshop Software. ImageJ Software was then used to hand-count c-Fos IR+ cells and the average number between the three boxes for

each region was determined. These procedures were conducted by investigators blind to experimental condition.

Results

Intra-DLS GPER1 activation attenuates cocaine-induced c-Fos IR+ cells in the dorsomedial striatum

G1 treatment decreased cocaine-induced c-Fos IR in the DMS **(**Figure 15 A). Specifically, cocaine increased c-Fos IR+ cells by 210% in cholesterol animals, but only by 44% in G1 treated animals. A two-way ANOVA found a significant treatment x drug interaction ($F_{1,20}$ = 4.391; p = 0.0491). Bonferroni's multiple comparisons test determined that among animals that received cholesterol intra-DLS, there were a greater number of c-Fos IR+ cells in animals that were given cocaine than saline (p = 0.0485). Among G1 treated animals, however, there were no significant differences between saline and cocaine animals (p > 0.9999). No main effects were observed in the two-way ANOVA.

Intra-DLS GPER1 activation enhances c-Fos IR+ cells in the Nucleus Accumbens Core and Shell

G1 treated animals had 814% more c-Fos IR+ cells in the NAcC than cholesterol treated animals (Figure 15 B). A two-way ANOVA indicated a main effect of intra-DLS treatment ($F_{1,20}$ = 14.91; p = 0.0010) but no main effect of drug condition and no significant interaction. Similarly, c-Fos IR+ cells in the NAcSh were 156% greater in G1 treated animals (Figure 15 C), as suggested by a main effect of intra-DLS treatment ($F_{1,20}$ = 4.664; p = 0.0431).

In the NAcSh, the two-way ANOVA also indicated a significant treatment x drug interaction ($F_{1,20}$ = 5.414; p = 0.0306). Bonferroni's multiple comparisons test determined that among cholesterol treated animals, there were 151% more c-Fos IR+ cells in cocaine versus saline exposed animals (p = 0.0118). However, among G1 treated animals, the quantity of c-Fos IR+ cells did not differ between cocaine and saline exposed groups (p > 0.9999).

Prelimbic cortex and lateral globus pallidus are not affected by intra-DLS GPER1 activation or cocaine exposure

Levels of c-Fos IR+ cells in the PRL (Figure 15 D) and GPe (Figure 15 E) were not affected by intra-DLS treatment or drug condition. A two-way ANOVA investigated each of these regions independently and revealed no main effects or interactions.

Intra-DLS GPER1 activation potentiates locomotor activity after cocaine administration

During the animal's initial exposure to testing chamber, they were naïve to treatment conditions (Figure 16 A). A two-way ANOVA indicated that, at baseline, there were no significant differences in total distance traveled between group assignments and could therefore be compared after drug/treatment administration to determine the effects of saline versus cocaine, cholesterol versus G1, and any potential interactions. On day 2, there were four distinct treatment conditions: saline + cholesterol, cocaine + cholesterol, saline + G1, and cocaine + G1 (Figure 16 B). There was a main effect of drug ($F_{1,20}$ = 36.30; p < 0.0001) but no main effect of intra-DLS treatment ($F_{1,20}$ = 3.564; p = 0.0736). Additionally, there was a significant interaction between drug and treatment conditions ($F_{1,20}$ = 4.783; p = 0.0408). Bonferroni's multiple comparisons test

determined among animals that received cocaine, those treated with G1 had significantly greater locomotor behavior than those treated with cholesterol ($p = 0.0185$).

Discussion

Our results support that intra-DLS GPER1 activation attenuates cocaine-induced neuronal activity in the DMS, supported by the attenuation of c-Fos IR. We also report that in the NAcC and NAcSh, intra-DLS GPER1 activation alone is sufficient to increase quantity of c-Fos IR+ cells to a degree equal to or greater than that induced by cocaine alone, and the GPER1-induced increase in c-Fos was not raised any further by adding cocaine to GPER1. However, we did not find an effect of cocaine or GPER1 activation on c-Fos IR in the prelimbic cortex or external globus pallidus. Finally, our results indicate that there are compound effects of cocaine and intra-DLS GPER1 activation on enhancing locomotor activity.

In the dorsal striatum, cocaine-induced c-Fos is mediated by D1 receptors and other have used induction of c-Fos as an indicator of D1 receptor signal transduction (Kim, Froelick, & Palmiter, 2002; Young, Porrino, & Iadarola, 1991). Our findings that G1 treatment attenuates c-Fos in cocaine-treated animals suggests that GPER1 activation is attenuating D1 receptor activation in the DMS (Figure 15 A). One possible mechanism that could be mediating this effect is that GPER1 activation may be is downregulating D1 receptor expression, accounting for the decreased neuronal activation after cocaine. Alternatively, GPER1 may be affecting the pre-synaptic dopamine transporter and causing an alteration in pre-synaptic dopamine reuptake, which would indirectly be affecting the amount of dopamine binding to D1 receptors. As discussed in the introduction, intra-DLS G1 administration attenuates males' cocaine

conditioned place preference. Given that the rewarding properties of cocaine are, in-part, due to the drugs' effects on enhancing synaptic dopamine transmission, we hypothesize that GPER1 activation is likely to be affecting dopamine neurotransmission and causing this attenuation in preference.

In both the NAcC and NAcSh, G1 treatment enhanced overall neuronal activation (Figure 15 B-C). The increase in the quantity of c-Fos+ cells were nearly identical in cocaine exposed cholesterol-treated animals and saline exposed G1-treated animals. Interestingly, G1 and cocaine together did not cause any greater c-Fos IR levels than either independent treatment suggesting that a ceiling effect may be occurring for the total amount c-Fos IR.

From this, we hypothesize that same population of cells are being activated by either cocaine or G1. The localization of GPER1 in the ventral striatum remains unclear. Previous work has shown the localization of GPER1 to GABAergic and cholinergic neurons in the dorsal striatum of females however, these studies did not investigate males (Almey, Filardo, Milner, & Brake, 2012; Almey et al., 2016).

We aimed to determine whether intra-DLS GPER1 alters patterns of c-Fos expression in distant brain regions that either project to or from the dorsal striatum. We determined that G1 treatment intra-DLS did not significantly alter neuronal activation in the PRL (Figure 15 D).This area was included because GPER1 receptors have been identified here and it is a region of striatal input to the DMS and NAcC (Hazell et al., 2009; McGeorge & Faull, 1989; Takahashi, Schoenbaum, & Niv, 2008). However, it is probably unsurprising that pharmacological manipulation did not cause a retrograde effect in neuronal activation.

We also hypothesized that GPER1 activation intra-DLS would alter activation of the indirect pathway. However, we found relatively few c-Fos IR+ cells in both of these regions overall, and these levels did not change significantly from cocaine or G1 treatment (Figure 15 E). This findings is in line with other' reports of relatively low cocaine-induced c-Fos in the indirect pathway (Cenci, Campbell, Wictorin, & Björklund, 1992). From this we cannot confidently determine whether GPER1 attenuates neuronal activation, due to the low number of c-Fos+ cells overall however, it appears that GPER1 activation does not increase activation within the GPe. Others have noted that amphetamine administration in a novel environment induces greater c-Fos in some regions of the indirect pathway (Uslaner et al., 2001). Animals in the current experiment were habituated to the chamber for one day prior to cocaine administration but perhaps future studies could repeat this in a novel context to better determine whether GPER1 activation intra-DLS attenuates downstream neuronal activation.

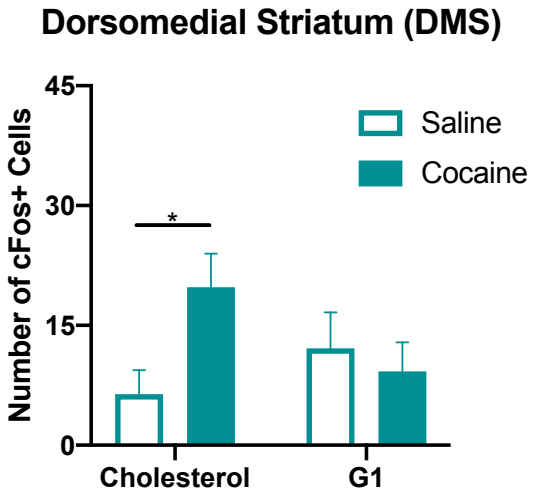
This is the first study to determine that estradiol may enhance acute sensitization to cocaine in males. Activation of D1 receptors in the NAc enhances behavioral activity in male rats (Dreher & Jackson, 1989). Above, we suggest that GPER1 activation intra-DLS is activating the same population of cells that are activated by cocaine therefore, the potentiation of locomotor activity could be D1 receptor mediated. However, significantly more evidence is needed prior to suggesting a causal effect.

While our results provide important and novel findings, there are significant limitations to the current study that should be addressed. To begin with, we have speculated the implications of intra-DLS GPER1 activation, but much more evidence is needed before any causal statement can be made. While the quantification of c-Fos

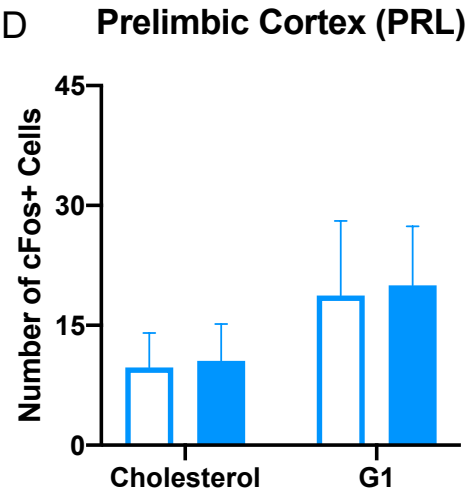
provides some information about which brain regions may be activated by treatment conditions, we do not know the mechanisms behind the changes in neuronal activation. Further, there are other immediate-early gene products that may also be involved in the neural response to GPER1 and cocaine that we did not capture by only looking at c-Fos. Additionally, we administered treatment stylets into the DLS however, this route of administration damaged tissue beyond being able to image or quantify it. Finally, this study did not include females and is significantly limited for that reason.

As shown in Figure 18, intra-DLS GPER1 activation caused the greatest changes in c-Fos expression in striatal regions, which were closest in proximity to stylet administration of G1. While this study has significant limitations, it does make clear that there are neural mechanisms being altered by the presence of intra-striatal G1 and more studies need to be conducted to investigate what these changes are, as these neural mechanisms are pertinent to understanding the recent findings that GPER1 may be neuroprotective in males' reward-circuitry.

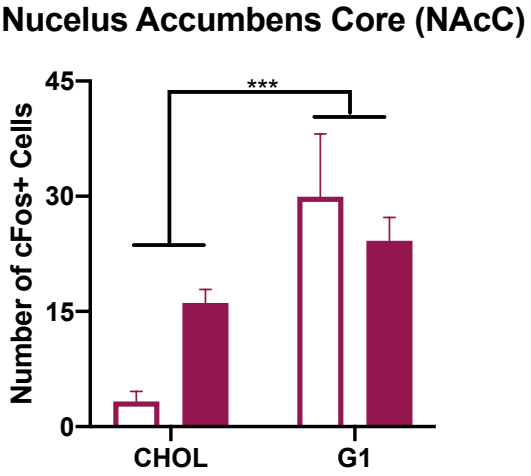
A


Dorsomedial Striatum (DMS)
Saline
Cocaine
Number of cFos+ Cells
45
30
15
0
*
Cholesterol
G1

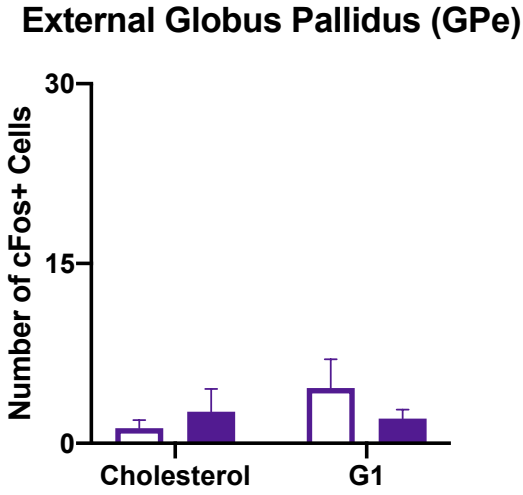
D


Prelimbic Cortex (PRL)
Number of cFos+ Cells
45
30
15
0
Cholesterol
G1

B


Nucelus Accumbens Core (NAcC)
Number of cFos+ Cells
45
30
15
0
***
CHOL
G1

E


External Globus Pallidus (GPe)
Number of cFos+ Cells
30
15
0
Cholesterol
G1

C

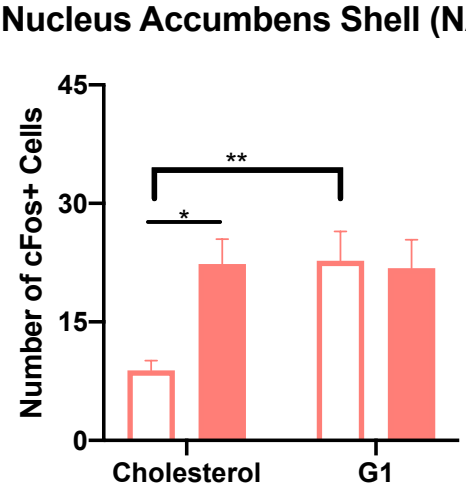
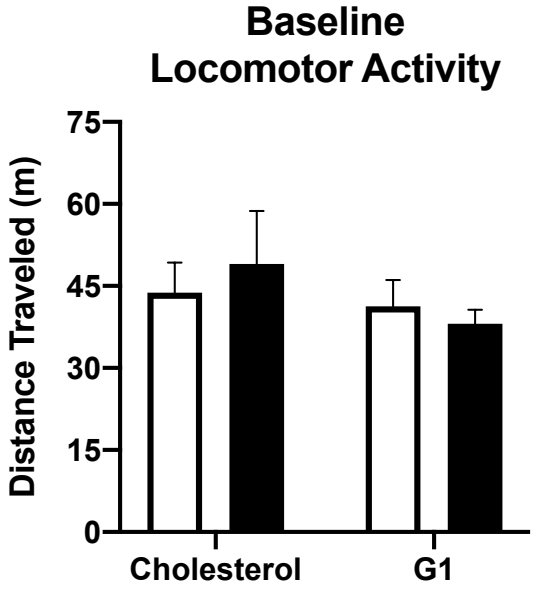

Nucleus Accumbens Shell (NAcSh)
Number of cFos+ Cells
45
30
15
0
**
*
Cholesterol
G1

Figure 15 c-Fos immunoreactivity induced by intra-DLS GPER1 activation.

(A) In the dorsomedial striatum (DMS), there were a greater number of c-Fos IR+ cells in cocaine versus than saline exposed ($p = 0.0485$) cholesterol treated animals, but no difference between G1 treated animals ($p > 0.9999$). (B-C) In the Nucleus Accumbens core (NAcC) and shell (NAcSh), G1 treatment enhanced the quantity of c-Fos IR+ cells ($p = 0.0431$; $p = 0.0306$). (E-F) Neither cocaine nor G1 treatment altered the quantity of c-Fos IR+ cells in the prelimbic cortex (PRL) or external globus pallidus (GPe). Data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

A


Baseline
Locomotor Activity
Distance Traveled (m)
75
60
45
30
15
0
Cholesterol
G1

B

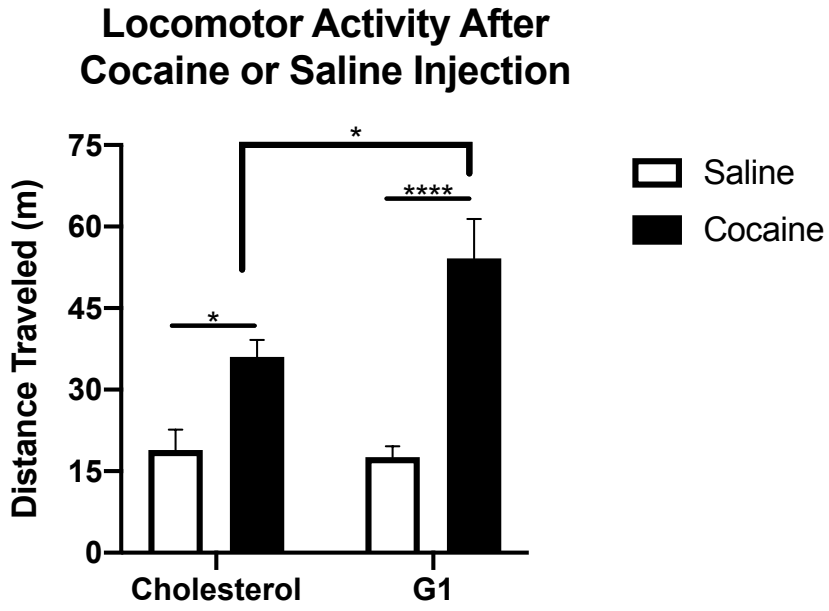
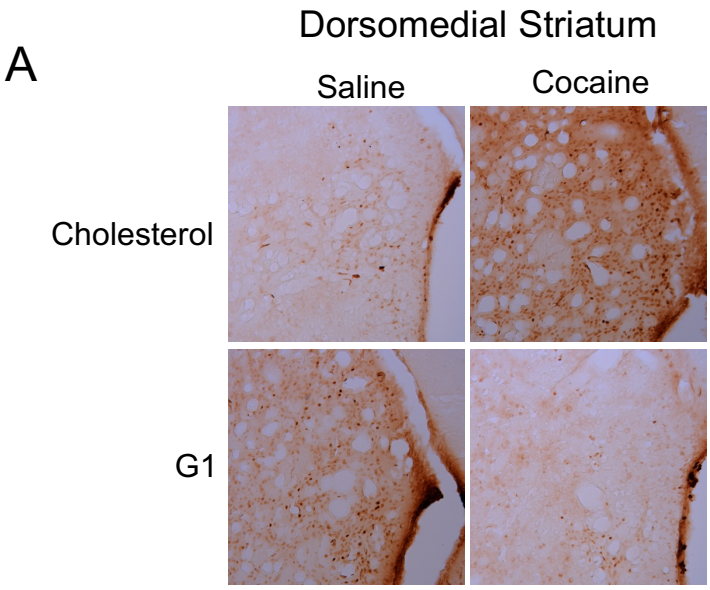
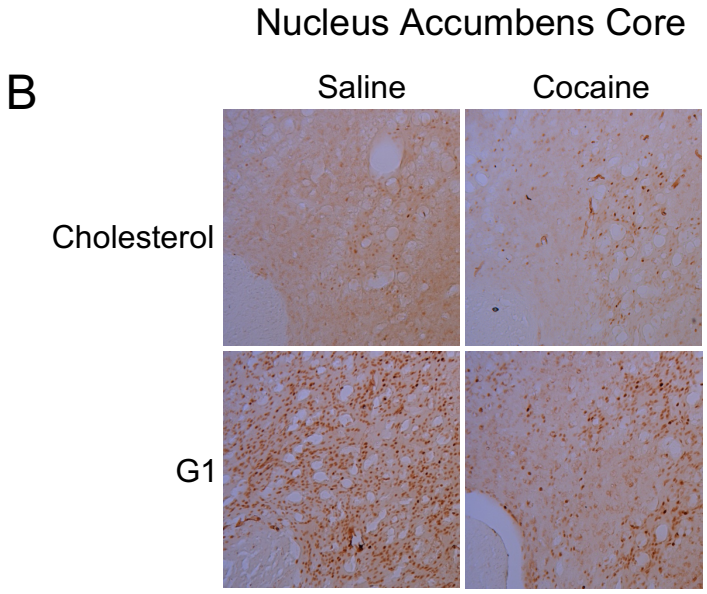

Locomotor Activity After
Cocaine or Saline Injection
Distance Traveled (m)
75
60
45
30
15
0
*
****
*
Cholesterol
G1
Saline
Cocaine

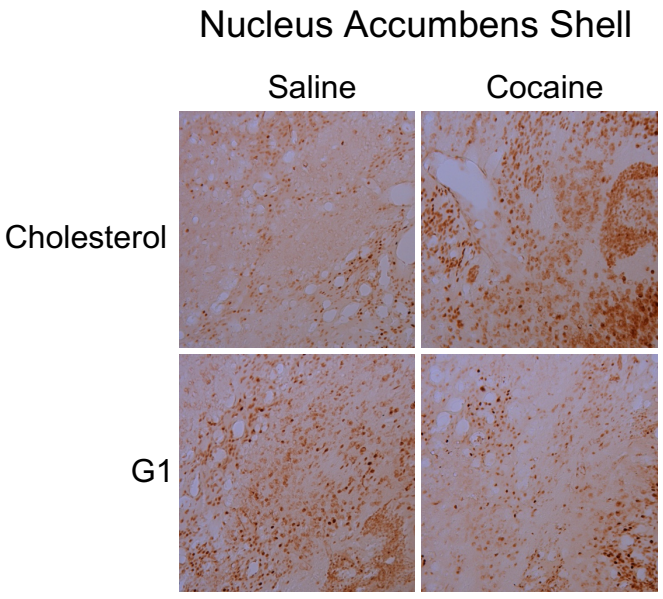
Figure 16 Alterations in locomotor activity after cocaine or GPER1 activation.

(A) At baseline, there are no significant differences in locomotor activity between groups, prior to cholesterol/G1 treatment or cocaine exposure. (B) Cocaine exposed animals have significantly greater locomotor activity than saline exposure animals. G1 treatment significantly enhanced locomotor activity compared to cholesterol treatment ($p = 0.0185$). Data are presented as mean ± SEM; $*p < 0.05$

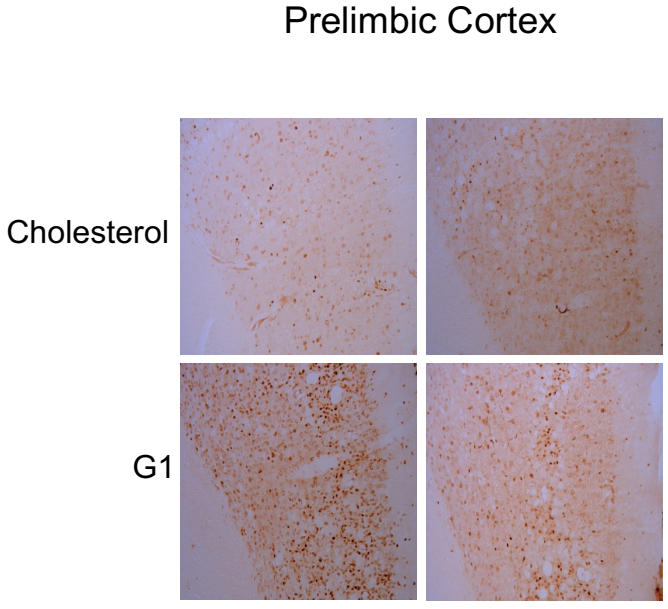

A
Dorsomedial Striatum
Saline
Cocaine
Cholesterol
G1


B
Nucleus Accumbens Core
Saline
Cocaine
Cholesterol
G1

C


Nucleus Accumbens Shell
Saline
Cocaine
Cholesterol
G1

D


Prelimbic Cortex
Cholesterol
G1

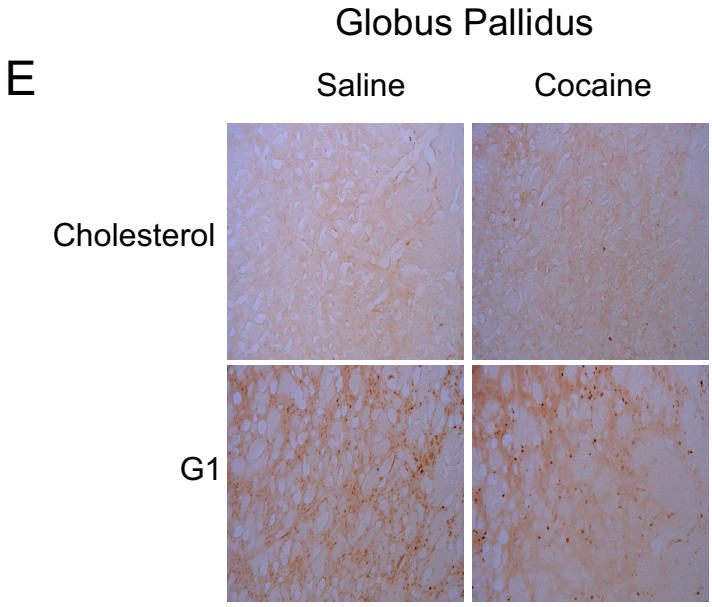

Globus Pallidus
E
Saline
Cocaine
Cholesterol
G1

Figure 17 Representative images of brain regions quantified for c-Fos immunoreactivity.

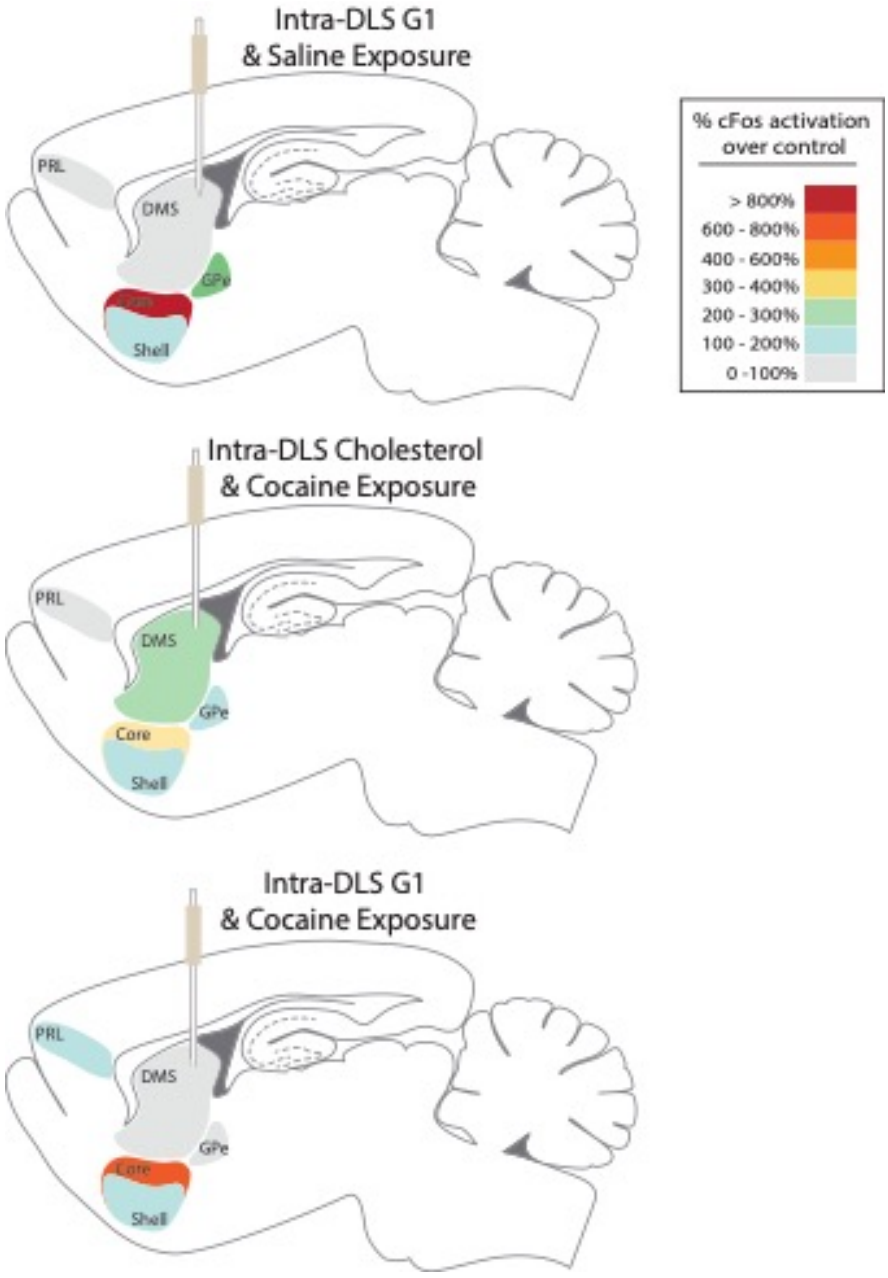

Intra-DLS G1
& Saline Exposure
PRL
DMS
GPe
Core
Shell
% cFos activation
over control
> 800%
600 - 800%
400 - 600%
300 - 400%
200 - 300%
100 - 200%
0 -100%
Intra-DLS Cholesterol
& Cocaine Exposure
PRL
DMS
GPe
Core
Shell
Intra-DLS G1
& Cocaine Exposure
PRL
DMS
GPe
Core
Shell

Figure 18 Schematic illustrating percent increase of c-Fos immunoreactivity.

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Chapter V
Discussion

Sex differences in addiction are influenced by the presence and function of estradiol in the brain. Estradiol enhances the rewarding properties of drug of abuse for females, which contributes to their increased motivation for drug-seeking (Yoest, Cummings, & Becker, 2014). Although estradiol is thought of as being a female centric gonadal hormone, it has important functions in the male brain as well. For example, testosterone is converted to estradiol via the aromatase enzyme and this presence of estradiol during fetal development is responsible for masculinization of the brain (McCarthy, 2008). During adulthood, testosterone continues to be aromatized to estradiol in the brain and via estradiol receptor (ER) subtypes: ERα, ERβ, and GPER1. These estradiol receptor subtypes are all present in the male brain at similar quantities to the female brain (Quigley et al., 2021). Despite the well-understood role of estradiol modulating reward in females, the role of estradiol in relation to drug-abuse liability for males, if one exists, has not been identified.

Synopsis

GPER1 modulates expression of preference drug and non-drug rewards in males

As discussed extensively in the introduction (Chapter I) of this dissertation, decades of clinical and pre-clinical research investigating the function of estradiol in substance abuse support that estradiol enhances drug abuse liability for females via classic estradiol receptors: ERα and ERβ. The extent to which estradiol influences the

propensity for drug seeking in males has been investigated to a lesser degree, primarily because initial studies which did include males did not find a behavioral or mechanistic role for estradiol in facilitating males' motivated behaviors, such as drug-seeking.

I began the research described in chapter II with the hypothesis that inhibition of estradiol receptors in the dorsolateral striatum (DLS) would decrease females' preference for cocaine and that there would be no effect in males. However, I found the opposite: administration of ICI 182,780 (ICI) intra DLS attenuated cocaine condition place preference (CPP) in males and had no effect in females. Initial studies reported ICI as an estradiol receptor antagonist ERα and ERβ, however, this was before the discovery of the orphan estradiol receptor, GPER1, at which ICI also acts as an agonist. This information led to me to investigate whether pharmacological activation of GPER1 would replicate the behavioral results of ICI.

In support of my hypothesis, I found that activating GPER1 intra-DLS was sufficient to attenuate cocaine CPP in males but not females. I further investigated the extent to which GPER1 could play a modulatory role in preference behavior by determining that inhibition of GPER1 could also enhance cocaine CPP, at a dose which otherwise does not produce conditioning effects. Together, this information led me to hypothesize that GPER1 is regulating the rewarding effects of drugs of abuse in males, but I remained curious to determine if GPER1 was also able to inhibit the preference for non-drug rewards as well.

Using a two-bottle choice paradigm, I assessed how intra-DLS GPER1 activation affects preference for 0.1% saccharin versus plain water. My results indicated that, similar to the effects of GPER1 on cocaine-preference, preference for 0.1%

saccharin solution was blocked for males but not females. After analyzing the amount of liquid consumed between groups and not findings any effect of the GPER1 agonist, I report that GPER1 activation was not causing an overall malaise in males. Instead, GPER1 is modulating reward by another mechanism for males and this effect is conserved across varying reward-inducing stimuli.

Finally, in this chapter I explored whether there are sex differences in estradiol receptor expression in the dorsal striatum, as possible explanation as to why pharmacological activation of GPER1 was causing changes in behavior for males but not females. Similar to what others have reported, I did not find any sex differences in relative mRNA levels of ERα, ERβ, or GPER1. Interestingly, I did find significantly greater levels of GPER1 mRNA compared to ERα and ERβ, for both sexes. This could be one potential explanation for why the effects of ICI, a nonselective ERα/β antagonist and GPER1 agonist, resulted in robust effects that align with the selective GPER1 agonist in males.

GPER1 enhances females' but not male's motivation to attain drugs of abuse and drug-induced reinstatement

A progressive increase in the intensity and frequency of drug use, along with heightened motivation to take drugs, are behavioral characteristics of addiction. While pre-clinical models of addiction can be limited in translational relevance in some respects, such as measuring the subjective feelings of drugs of abuse, they also very consistently capture other aspects, such as the enhancement of motivation to attain drugs over time. For chapter III, I chose to use a self-administration model to determine whether intra-DLS GPER1 activation alters motivation to attain cocaine, post-

acquisition, in either sex. I also investigate the extent to which GPER1 activation alters drug-induced reinstatement in these animals.

Based on findings from chapter II, I expected to see that activation of intra-DLS GPER1 would inhibit motivation for cocaine in males. However, my results do not support this hypothesis. Instead, I found that males in both treatment groups showed similar increases in motivation for cocaine over time. Additionally, GPER1 activation did not appear to influence drug-induced reinstatement in males. The caveat in this study is that the GPER1 agonist was administered after animals had already acquired cocaine-seeking behavior. It is possible that activation of the GPER1 receptor intra-DLS prior to exposure to cocaine would have been more effective in attenuating both acquisition and overall motivation.

In chapter III, I report that GPER1 activation causes an increase in motivation for cocaine in females as well as enhance drug-induced reinstatement. This finding is consistent with prior work which has established that the presence of estradiol increases self-administration of psychostimulants for females. However, my data are the first study to identify that GPER1 receptors intra-DLS specifically, can cause enhanced motivation in females. It is worth noting that ERα and ERβ are also localized in the DLS and because the females in my study are intact, some circulating estradiol is likely binding and activating these receptors as well.

Alterations in c-Fos immunoreactivity after pharmacological activation of GPER1

The goal of chapter IV was to explore how intra-DLS GPER1 activation alters neuronal activation in the brains of naïve and cocaine-exposed animals. I quantified levels of c-Fos immunoreactivity (IR) in subregions of the striatum including the

dorsomedial striatum, nucelus accumbens core, and nucelus accumbens shell. I also quantified regions which project to and from the DLS: the prelimbic cortex and external globus pallidus, respectively. Because very little research has gone into identifying the neurobiological mechanisms of GPER1 activation in males, the goal of this exploratory experiment was to give insight on what brain regions or circuits might be implicated in regulating the drug-preference shifts and alterations in motivation reported in chapters 2 and 3. The major limitation to this chapter is that only males were included, and future iterations of this study should explore females.

The effects of GPER1 activation alone, cocaine exposure alone, or GPER1 activation in cocaine exposed animals all produced different effects on c-Fos IR that was dependent on subregions of the striatum. In the dorsomedial striatum, cocaine enhanced neuronal activation but GPER1 attenuated this effect. In the nucleus accumbens core and shell subregions, cocaine alone or intra-DLS GPER1 activation alone enhanced neuronal activation to a similar extent however, there were no compound effect of the two treatments on c-Fos IR levels. No effects were observed in the prelimbic cortex or external globus pallidus. We infer from these results that the dorsal and ventral regions of the striatum should be examined mechanistically for alterations caused by GPER1 activation. Hypotheses for these mechanisms as well as ways in which this can be explored are outlined below.

Types of sex differences in animal models of addiction

Sex differences observed from animal models of addiction can be categorized into qualitative differences, quantitative differences, population differences, as well as differences in underlying mechanisms between males and females (Becker & Koob,

2016). Often, the underlying neurobiology of males and females cause mechanistic differences that perpetuate sexually dimorphic behaviors. Drugs of abuse also change neurocircuitry differently in males versus females, which contributes to further sex-dependent changes in motivation and decision making.

Quantitative sex differences are exemplified by magnitude of behavior response differing between males and females. In chapter II, the magnitude of preference of cocaine is greater for males than females and females' motivation for cocaine is greater than males, as reported in chapter III. These studies were designed so that there were not sex differences at baseline response for drug. Manipulation of striatal GPER1 altered response for cocaine in either sex in different directions, suggesting that the underlying mechanisms by which GPER1 regulates reward and behavior are different between males and females. Below are proposed sex-dependent mechanisms by which striatal GPER1 may be mediating reward.

Proposed role of estradiol and GPER1 in females

In females, estradiol enhances cocaine-induced dopamine release in the dorsal striatum (Becker, 1990; Yoest, Cummings, & Becker, 2019). Dopamine is important for attributing incentive salience to reward-related stimuli and mediating the pursuit of reward (Berridge, 2007). Therefore, dopamine transmission after estradiol treatment is linked to females' enhanced propensity to self-administer cocaine (Martinez et al., 2016). Previous behavioral studies were estradiol replacement studies and did not investigate the contribution of specific estradiol receptors in mediating motivation in females. The results from chapter III indicate activating GPER1 specifically is sufficient

to alter behavior. I hypothesize that this is likely due to an enhancement in cocaine-induced dopamine, induced by GPER1 activation in the DLS.

I did not find that GPER1 activation alters cocaine CPP for females, which I speculate could be for multiple reasons. First, these results may suggest that estradiol is important for facilitating motivation more so than reward-preference in females. However, others have shown that estradiol enhances cocaine conditioned place preference, but these were in ovariectomized female rats (Bobzean, Dennis, & Perrotti, 2014; Russo et al., 2003). Secondly, there is evidence to suggest that, for females, the rewarding properties of cocaine are mediated by ERβ in the ventral striatum (Satta, Certa, He, & Lasek, 2018). It may be that activation of ERβ simultaneous to GPER1 is necessary to induce behavioral changes in females.

Finally, the lack of significant effect of GPER1 activation on cocaine CPP or saccharin preference in females could be due to a ceiling effect in the behavioral paradigms used to assess reward preference. Both the cocaine conditioned place preference and saccharin two-bottle choice studies used doses which provoke a preference in females. If a lower dose, that does not provoke a place preference of cocaine were used, I hypothesize that intra-DLS GPER1 activation may have induced a greater effect and enhanced cocaine CPP in females.

Future directions

An important next step to understanding the role of GPER1 in females is to investigate the effects of GPER1 on striatal dopamine levels. In females, most ERα and GPER1 are localized to GABAergic medium spiny neurons in the striatum (Almey, Milner, & Brake, 2016). Effects of ERα inhibition on GABAergic interneurons indirectly disinhibitions

dopaminergic neurons and increases stratal dopamine levels. I hypothesize that based on the location of GPER1 receptors, a similar mechanism is at play.

Proposed role of estradiol and GPER1 in males

In males, I report that GPER1 activation attenuates cocaine-induced c-Fos IR in the dorsomedial striatum. Generally, cocaine enhances striatal c-Fos levels via dopamine D1 receptor activation. Given this, I hypothesize that the attenuation of c-Fos immunoreactivity, after GPER1 activation, could be due to an attenuation of D1 receptor activation (Young, Porrino, & Iadarola, 1991). For a decrease in D1 receptor activity to occur, there is likely less dopamine transmission in this region, overall. Because dopamine is involved in the attribution of salience to reward-related stimuli, a reduction in striatal dopamine could be a cause for males not showing a preference for drug-associated environment, such as shown in chapter II.

As to be expected, cocaine enhanced c-Fos IR in the nucleus accumbens. Interestingly, the GPER1 agonist also caused robust neuronal activation in the nucleus accumbens and to similar levels as cocaine did. However, the two treatment together, cocaine and GPER1 agonist, did not have any compound effects on c-Fos levels, suggesting that these different stimuli may be activating the same populations of neurons in the nucleus accumbens. Certain subpopulations of neurons, primarily distinguished by the rostral and caudal shell, regulate reward and activation of these populations causes intense liking or aversive responses. As shown in chapter II, males show a slight aversion to saccharin after intra-DLS GPER1 treatment. I hypothesize that GPER1 activation in the dorsal striatum may indirectly be acting subpopulations of neurons in the ventral striatum and contributing to the aversiveness of saccharin.

Future directions

The robust effects of GPER1 on reward preference in males do not translate to altering motivation, in my studies. To rule out that GPER1 mediates motivation for males altogether, a reasonable next step is to give the GPER1 agonist prior to acquisition of cocaine self-administration. If there is still no effect on motivation, then the outcome from this study would educate future directions for studying liking versus wanting and the associated pathways of the brain. Alternatively, if GPER1 activation blocks acquisition of self-administration behavior this would suggest that GPER1 may be mediating learning and reward-associations.

Before we can theorize how GPER1 is acting to decrease the value of rewarding stimuli in males, it is pertinent that we understand how activation of GPER1 is altering dopamine transmission in the dorsal and ventral striatum. Future studies need to determine the effects of DLS GPER1 activation on basal and drug-induced dopamine levels. An equally important piece of this puzzle, and an important next step, is to investigate what neuron types GPER1 is localized to throughout the striatum because no studies have looked in males.

Conditioned place preference encompasses multiple psychological aspects including incentive-driven behavior, conditioned treatment effects, and the learning reward-context association (Huston, Silva, Topic, & Müller, 2013). I showed that intra-DLS GPER1 manipulation decreases or increases cocaine condition place preference in males however, the psychological construct that is affected to illicit these behavioral changes is not fully clear. Recent evidence implicates GPER1 in learning and memory function in females, but this has not been explored in males (Kim et al., 2019; Kim,

Szinte, Boulware, & Frick, 2016). Future studies investigating pharmacological activation of GPER1 at different timepoints of conditioned place preference conditioning would help to decipher if these are learning effects in males.

Other considerations and future directions

In the studies presented here, male and female rats remained gonadally intact and endogenous hormone levels were not measured or controlled. Therefore, at some level, ERα and ERβ are likely being activated by endogenous estradiol levels. Previous studies have mainly used methods of gonadectomy and hormone replacement to investigate the role of estradiol for males and females. One issue with these replacement studies is that estradiol receptors in the brain decrease in quantity in the absence of estradiol. Whether there are sex differences in the rate of estradiol receptor downregulation has not been investigated and is an avenue for future research.

At the cellular level, GPER1 are localized to the extracellular membrane and also on the membrane of endoplasmic reticulum (Otto et al., 2008; Zimmerman, Budish, Kashyap, & Lindsey, 2016). Cyclic-AMP and calcium are often the messengers associated with GPER1 signaling but there are many others suggested to play a role, and these are dependent on cell-type being studied (Nilsson, Olde, & Leeb-Lundberg, 2011). While outside of the scope of this dissertation, understanding the effects of GPER1 on downstream signaling cascades is certainly an important goal for future research.

Clinical relevance

A fundamental next step for clinical scientists is to identify how estradiol may be playing a role in human's propensity for addiction. As discussed extensively in chapter I,

estradiol is seen to enhance vulnerability for escalation of drug use, craving and relapse in women. However, there is little to no literature on the protective effects of estradiol in males. In this set of studies, I have shown that activating GPER1 decreases the value of cocaine for male rats. I hypothesize that estradiol, possibly via GPER1, may have similar effects in humans implying that not only does estradiol enhance vulnerability in females, but may be protective in males. I suggest that GPER1 and drugs that target this receptor be studies and treated as a potential neuronal target for the treatment of addiction in males. There are selective estradiol receptor modulators currently approved for use in clinical setting. One such drug is Raloxifene, a selective GPER1 agonist in the brain that does not cause estrogenic effects in the periphery.

Conclusions

Substance abuse and addiction to illicit drugs destroys lives. It is necessary that we continue to study the biological basis for addiction to get closer to effective and sustainable treatment outcomes. Pre-clinical models are an exceptional source to understanding what variables influence drug-seeking in specific populations, including sex differences. Here, I present a series of experiments investigating the contribution of a specific estradiol receptor subtype, GPER1, in mediating sex differences observed in addiction vulnerability and reward. While more research is needed to confirm this, it appears that GPER1 may be protective against drug-reward is males and may be a target for therapeutic treatment for addiction in males. This set of studies also highlights the importance of basic-science laboratories using the right behavioral measures to study males and females, which may not always be the same. Further, I show an effect of GPER1 in males but not females in chapter II, and the opposite in chapter III,

indicating that males and females cannot always be studied and compared as if their underlying neurobiology are the same. It is always necessary to include both sexes, which I urge future research to do.

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