ABSTRACT

PROAÑO, STEPHANIE BETSAIDA. Estrous Cycle and Ovarian Hormone Effects on Nucleus Accumbens Core Medium Spiny Neuron Physiology. (Under the direction of Dr. John Meitzen and Dr. Heather Patisaul).

Naturally occurring cyclical changes in sex steroid hormones, such as estradiol and progesterone, can modulate brain function and behavior in spontaneously ovulating female animals, such as rats and humans. In rats, the cycle is called the estrous cycle and is comparable to the human menstrual cycle. The estrous cycle features cyclical changes in ovarian hormones, such as estradiol and progesterone, and is divided into distinct phases. These phases are characterized by differing concentrations of estradiol and progesterone as well as by accompanying changes in reproductive organ physiology and associated reproductive behaviors. In addition, hormone fluctuations across the estrous cycle induce sex- and estrous cycle-dependent differences in the phenotypes of many behavior and disorders outside of reproductive contexts. These behaviors and disorders include those related to motivation, reward, and reinforcements such as anxiety, depression, and addiction. This suggests that the neural substrate instrumental for these behaviors, including the nucleus accumbens core (AcbC), likewise differs by sex and between estrous cycle phases. The AcbC is a well conserved brain region between rodents and humans and functions as the nexus between the limbic and pre-motor systems to process the cognitive and motor output related to motivation, reward, and reinforcement. It is unknown if the electrophysiological properties of AcbC output neurons, medium spiny neurons (MSNs), change across the estrous cycle and if these changes are coordinated by independent or combined action of estradiol and progesterone. This is a significant knowledge gap given that AcbC MSN electrophysiological properties determine the ultimate output from this brain region, thus underlying the expression of AcbC-mediated behaviors and disorders that differ by

biological sex and between estrous cycle phase. Here I comprehensively test the differential expression of AcbC MSN excitatory synapse and intrinsic excitability properties across all phases of the estrous cycle and under the influence of estrous cycle relevant doses of estradiol (17beta estradiol and estradiol benzoate) employing whole-cell patch clamp electrophysiology. In addition, I investigate the association between circulating levels of estradiol and progesterone across the estrous cycle with AcbC MSN excitatory synapse and intrinsic excitability properties employing a hormone extraction technique. By employing these methods I find that: 1) estrous cycle phase robustly modulates AcbC excitatory and intrinsic excitability properties; 2) removal of the estrous cycle via gonadectomy abolishes sex- and estrous-cycle dependent differences in AcbC MSN excitatory synapse and intrinsic excitability properties; 3) estradiol and progesterone differentially and synergistically influence the electrophysiology of AcbC MSNs, with estradiol mostly modulating intrinsic excitability properties and progesterone primarily modulating excitatory synapse properties; and 4) estrous cycle relevant doses of estradiol administered to mimic the natural fluctuations of estradiol across the estrous cycle recapitulate the decrease in intrinsic excitability observed in naturally cycling female rats during the proestrus AM and estrus phases of the cycle. These findings are the first to elucidate estrous cycle and ovarian hormone effects on the input/output properties of AcbC MSNs. In a broader context, these findings provide the first line of evidence for the underlying neuroendocrine mechanisms behind sex- and estrous cycle dependent differences in AcbC-mediated behaviors and disorders.

Estrous Cycle and Ovarian Hormone Effects on Nucleus Accumbens Core Medium Spiny Neuron Physiology

by
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DEDICATION

To my mom, Azucena Rivera. With you I can do anything.

IN MEMORIAM

To Ms. Edi and Mr. Rusty Lyles. You made my beginnings in this country joyful.

BIOGRAPHY

Stephanie Proaño was born in Quito, Ecuador, where she lived until her high school junior year. At this time, she moved to Murfreesboro, a quaint small town in northeastern North Carolina. Here, she spent many formative years as she navigated the transition between adolescence into adulthood while trying to get accustomed to a new environment. It was during this time that she became fascinated by the human body and the potential of a career in medicine after taking a health class. This led her to pursue a major in Biological sciences once she started college with the aspiration to go to medical school upon completion of her degree. However, two important events took place as she pursued her undergraduate studies. First, a general education class requirement led her to take an introductory sociology class that forever changed her view of the world and made her add Sociology as a second degree. Second, during her final year in college, she took a fateful neurobiology course that sparked her interest in neuroscience and her love for the brain. These two events led her to delay going to medical school so that she could immerse herself in neuroscience research by joining the Meitzen laboratory at North Carolina State University. Little did she know that this move would completely change her professional trajectory.

Upon starting in the Meitzen lab, and even two years into her work there, Stephanie still had her eyes on medical school. She was very interested in understanding the inner workings of the brain, but she was also pulled by medical school and the opportunity to apply both her scientific and sociology backgrounds to treat patients. However, as her research progressed, Stephanie realized that investigating the brain was not only a fascinating endeavor that sparked her curiosity but also one that filled her life with purpose. Understanding the brain, she came to realize, also gave her the opportunity to advance the human condition as she once hoped to do as

a doctor. In addition, her work on the interplay between ovarian hormone cycles and the brain has been an avenue to apply her knowledge of sociology to push for the inclusion of female animals in basic neuroscience research so that better tools, treatments, and knowledge regarding female physiology become available. Upon completion of her doctoral degree, Stephanie will join the Dudek lab at the National Institute of Environmental Health Sciences at the National Institute of Health. Here, she hopes to continue learning about the brain as well as extend her advocacy for the investigation of female physiology.

When Stephanie is not within the trenches of science, she enjoys reading spy novels, listening to music (a lot of Heavy Metal), spending time with her partner, friends and family, and hiking along the trails of the US's most iconic national parks and forests.

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I want to thank Dr. Jean King, Mama King. Thank you for caring so much about me and my well-being. You were critical in the journey that has led to this day and I'm not sure I would

be where I am today had it not been for you. I thank the universe every day for bringing us together at the MBL. You have been one of my strongest cheerleaders and that is something that means the world to me and does not go unnoticed in my life.

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CHAPTER 1

Introduction

The pattern of gonadal steroid hormone production in adult animals of reproductive age is one of the most compelling sex differences in animal physiology. Examples of these patterns include the menstrual cycle in humans and the estrous cycle in rats. This female-specific pattern of hormone production not only influences various physiological processes relevant for reproduction, but also influences brain function and behavior. How the estrous cycle influences function in a specific neuron type and brain region, the medium spiny neuron in the nucleus accumbens core, is the central question to my dissertation. To arrive at how I investigated this question, I must first detail the developmental events that give rise to the trajectory that results in estrous-like patterns of hormone production.

Brain sexual differentiation and establishment of the ovarian cycle

Sexual differentiation

Sexual differentiation begins early in development and requires the interplay of genetic, hormonal, and environmental factors (Balthazart 2011, Forger 2018, Grgurevic & Majdic 2016, McCarthy 2008, McCarthy 2019, McCarthy et al 2009, Nugent et al 2011, Schwarz & McCarthy 2008). In mammals, this process starts when sex chromosomes are paired and determine whether an organism will be genetically female or male. Organisms with XX chromosome pairing are genetically female, whereas organisms with XY chromosome pairing are genetically male (Turano et al 2019). At first, the gonads and internal reproductive organs of genetic females and males are undifferentiated and feature two sets of ducts, the müllerian and wolffian ducts. In XY organisms, the sex determining region Y (Sry) gene on the Y chromosome encodes for testis determining factor (TDF). TDF induces the undifferentiated gonads to become testes rather than

ovaries (Fechner 1996, Kashimada & Koopman 2010, Sekido 2014, Wilson & Davies 2007). Once the gonads develop into testes, they produce two hormones that are important for male development and male sexual differentiation: anti-müllerian hormone and testosterone. Anti-müllerian hormone causes the regression of the müllerian ducts and the transformation of the wolffian ducts into male internal reproductive organs. Simultaneously, testosterone production further facilitates the masculinization of several body parts that include the brain (Rey 2005). In contrast, the absence of anti-müllerian hormone and testosterone in genetic females promotes the regression of the wolffian ducts and the transformation of the müllerian ducts into female internal reproductive organs (Becker 2002, Whitehead 2001). Additionally, the lack of these hormones feminizes several body parts, including the brain.

Organizational/aromatization hypothesis of brain sexual differentiation: establishing the neural basis for ovulatory capacity

Sexual differentiation of the brain is closely linked to testosterone production during pre- and post-natal sensitive windows as well as by its aromatization into estradiol. Early groundbreaking experiments established that pre- and post-natal surges of testosterone in male rodents masculinized sexual behavior. Phoenix and colleagues showed that prenatal testosterone exposure in female guinea pigs inhibited female typical sexual behavior (i.e. lordosis) upon estradiol and progesterone exposure in adulthood (Phoenix et al 1959). This discovery served as the foundation that inspired the organizational hypothesis of brain sexual differentiation. The organizational hypothesis proposes that early testosterone exposure in male rodents alters the developing brain so that it functions in a masculine manner in adulthood (Arnold 2009). However, how testosterone masculinized/defeminized the brain was unknown until later experiments showed that testosterone is aromatized into estradiol by the enzyme aromatase

(Naftolin & Ryan 1975). Through this process, local estradiol action in the brain permanently organizes the neural substrate to later support male-specific behaviors (MacLusky et al 1987, McEwen et al 1977, Phoenix et al 1959). In females, however, the absence of pre- and post-natal testosterone surges prevents the masculinization of the central nervous system. This has important consequences for the sustainability of ovulation by the hypothalamic-pituitary-gonadal (HPG) axis in adult females. Pioneering experiments in rats showed that the absence of pre- and post-natal testosterone surges in females is necessary to promote ovulatory capacity. In a series of experiments, Carol Pfeiffer demonstrated that testicular testosterone secretion during the first few days of life determines whether a rat would have ovulatory capacity in adulthood. Adult male rats castrated shortly after birth were able to support ovulation upon ovary implantation. Conversely, female rats that received subcutaneous grafts of testicular tissue could not support ovulation as adults. Further, a single injection of testosterone during the first week of life in female rats prevented ovulation as adults (Pfeiffer 1936). These findings revealed that ovulatory capacity was organized early in life by the absence of pre- and post-natal testosterone exposure. However, the mechanisms by which this process directly affected the brain remained unanswered until it was proven that communication between the hypothalamus and the pituitary gland was essential to induce ovulation in females. Indeed, Geoffrey Harris showed that the hypothalamus orchestrates the function of the pituitary gland to promote ovulation. Surgical implantation of adult male rat pituitary gland onto adult female rat hypothalamus resulted in the male's ability to support ovulation, thus proving that the female hypothalamus had been permanently altered to support ovulation (Harris 1964). However, before the female hypothalamus can direct the neural and endocrine mechanisms necessary for ovulation to occur

upon the onset of puberty, it needs to be activated by the coordinated action of hypothalamic, pituitary, and gonadal hormones.

Activational hypothesis of brain sexual differentiation: activating the neural basis underlying ovulatory capacity

The activational effects of gonadal hormones were first observed when different combinations of gonadal hormones (i.e. estradiol and progesterone) and gonadal status (i.e. intact, ovariectomized, castrated) were paired to induce/inhibit lordosis. Normally, naturally cycling female rodents (and other mammals) exhibit lordosis, an arching of the back, to signal sexual receptivity and facilitate intromission by males (Flanagan-Cato 2011, Harlan et al 1984, Pfaff et al 2018, Pfaff et al 2008). In rats, ovariectomy results in an inability to exhibit lordosis. However, this behavior can be rescued by a series of estradiol injections followed by a single progesterone injection (Boling J.L. 1939, Rubin & Barfield 1983a, Rubin & Barfield 1983b, Whalen 1974). Interestingly, when the same hormone regimen is administered to castrated males, they display little to no lordosis behavior, or higher doses of estradiol and progesterone are required (Davidson 1969, Moreines et al 1986, Södersten 1976). This not only shows that the neural substrate that supports this specific behavior differs by sex but that gonadal hormones, in this case estradiol and progesterone, activate previously sexually differentiated circuits underlying this specific behavior. Important to the establishment of the ovarian cycle in females, estrogens and progesterone, along with the concerted action of hypothalamic and pituitary hormones, activate the previously feminized neural circuits that underlie the establishment of the ovarian cycle and ovulation that start at puberty.

The onset of puberty is marked by increased luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion by the pituitary gland (Whitehead 2001). This promotes

sexual maturity and leads to reproduction capacity. LH and FSH secretion is regulated by gonadotropin releasing hormone (GnRH), whose release is activated by kisspeptin in the hypothalamus (Sisk & Foster 2004, Skorupskaite et al 2014, Smith & Clarke 2007). Once GnRH is activated, it induces the secretion of LH and FSH. LH and FSH secretion primarily target the gonads and causes them to produce sex steroids such as estrogens, progesterone, and testosterone. The ovaries produce progesterone and estrogens; with estrogens referring to estradiol, estrone, and estriol. The testes, on the other hand, produce testosterone. Although both females and males have the ability to produce all of these hormones, the quantities and patterns of production differ between the sexes. Indeed, the pattern of ovarian hormone production in females is cyclical, divided into phases, and requires the delicate interplay of the hypothalamus, the pituitary, and the ovaries.

Ovarian Hormone Cycle

The ovarian cycle begins when females reach sexual maturity and the hypothalamus releases GnRH under the direction of kisspeptin (Sisk & Foster 2004, Skorupskaite et al 2014, Smith & Clarke 2007). GnRH activates the release of LH and FSH by the pituitary gland. LH and FSH, in turn, induce the production of estrogens and progesterone as well as the release of the oocyte by the ovaries. The production of estrogens and progesterone and the accompanying release of the oocyte occur in a cyclical pattern in both humans and rats. In females, this cyclical pattern is called the menstrual cycle, and in rats, it's called the estrous cycle. In humans, the menstrual cycle is ~28 days and is divided into the follicular and luteal phases. The follicular phase features slowly increasing levels of estradiol until peak levels are reached. This increase in estradiol, in turn, produces the LH surge that induces ovulation around day 14. Ovulation marks the onset of the luteal phase which features peak progesterone levels with some remaining

estradiol production (figure 1.1). At the end of this phase, hormone levels drastically decrease giving rise to menstruation (Barsom et al 2008, Sherman & Korenman 1975, Zuckerman 1949). In rodents, the fluctuation of estradiol and progesterone is similar, but the cycle is compressed into ~4-5 days and is tightly regulated by the light-dark cycle. The first phase of the estrous cycle is diestrus. During this phase, the hypothalamus begins secreting GnRH and, as a consequence, the pituitary starts secreting LH and FSH. This causes the ovary to begin follicular development and estradiol and progesterone production. The next phase in the estrous cycle is proestrus. This phase features peak levels of estradiol in late morning (Proestrus AM) and peak levels of progesterone in the late afternoon (Progesterone PM) (Adams et al 2018). In the morning, as estradiol levels rise, the hypothalamus increases its production of GnRH and the pituitary increases secretion of LH. Then, in the afternoon, about 4-6 hours after the estradiol peak, progesterone and LH levels significantly increase and peak. Once LH and progesterone are released into circulation, they target the mature follicle and induce ovulation 10-12 hours later. The final phase of the estrous cycle is estrus (Cora et al 2015, Hubscher et al 2005). This phase features decreased levels of estradiol and progesterone but marks the beginning of the sexually receptive period (figure 1.1). Although the cyclical pattern of ovarian hormone production in females directs physiological and behavioral processes relevant to reproduction, increasing evidence suggests that ovarian hormones also influence brain function in areas not necessarily linked with reproduction.

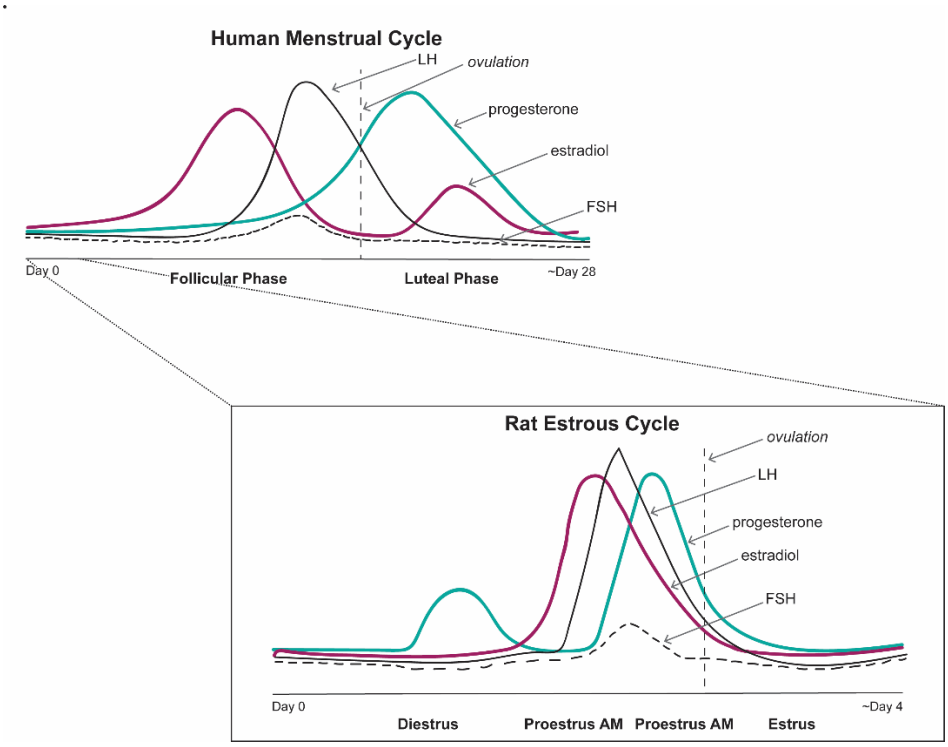

Figure 1.1. Human menstrual cycle and rat estrous cycle. The menstrual and estrous cycles feature cyclical fluctuations of ovarian hormones such as estradiol and progesterone. The menstrual cycle is divided into the follicular and luteal phases and spans over a period of about 28 days. The estrous cycle is divided into the diestrus, proestrus (AM and PM), and estrus phases and spans over a period of about 4 days.

Estrous cycle modulation of nucleus accumbens core structure and function

Nucleus accumbens core: Sex- and cycle-dependent differences in anatomy, circuitry, and function

The striatum is one such brain region that is not directly involved in reproduction but has shown sensitivity to the influence of biological sex and the cyclical fluctuations of ovarian hormones across the estrous cycle (Ikemoto & Panksepp 1999, Palmiter 2008, Stephenson-Jones et al 2011, Yoest et al 2018). The striatum is a well-conserved brain region across species that is implicated in the processing of motor and reward-related behavioral output (Burton et al 2015, Kreitzer & Berke 2011, Kreitzer & Malenka 2008). In rodents, the striatum is further subdivided into three regions that differ in afferent/efferent circuitry (Groenewegen et al 1999, Kreitzer & Berke 2011, Lynd-Balta & Haber 1994, Voorn et al 1989, Voorn et al 2004). These regions are

the nucleus accumbens core (AcbC), the nucleus accumbens shell (AcbS), and the caudate-putamen (CP). Although afferent inputs to and efferent outputs from these three regions differ, the predominant output neuron type is the same. GABAergic medium spiny neurons (MSNs) are the predominant neuron subtype, comprising about 95% of the entire neuronal population, from all three striatal subregions and receive dopaminergic, glutamatergic, and GABAergic inputs (Groenewegen et al 1999, Kreitzer & Berke 2011, Kreitzer & Malenka 2008, Lynd-Balta & Haber 1994, Palmiter 2008, Voorn et al 1989, Voorn et al 2004). Recent evidence has shown that there are sex- and estrous-cycle dependent differences is dopaminergic (Arnauld et al 1981, Becker 1990, Becker 1999, Becker et al 1984, Becker & Rudick 1999, Bonansco et al 2018, Cabrera & Navarro 1996, Calipari et al 2017, Czoty et al 2009, Di Paolo et al 1985, Tonn Eisinger et al 2018a, Yoest et al 2018), glutamatergic excitatory synapse markers (Cao et al 2016, Cao et al 2018, Forlano & Woolley 2010, Krentzel et al 2019, Peterson et al 2016, Peterson et al 2015, Staffend et al 2011, Wissman et al 2012, Wissman et al 2011), and MSN intrinsic excitability (Alonso-Caraballo & Ferrario 2019, Dorris et al 2015, Willett et al 2019) properties in these regions. Here, I will focus on sex- and estrous-cycle dependent differences in the AcbC given that this is the striatal subregion on which my dissertation is based.

The AcbC is a critical nexus region between the limbic and premotor systems that is involved in the cognitive processing of motor function related to motivated behaviors, reward, and reinforcement (Floresco 2015, Ikemoto & Panksepp 1999, Salgado & Kaplitt 2015). The AcbC receives glutamatergic inputs from pre-limbic and prefrontal cortices, amygdala, and hippocampus, and dopaminergic inputs from the ventral tegmental area (VTA) (Berridge & Kringelbach 2008, de Jong et al 2019, Park et al 2019, Salamone 1994, Salamone & Correa 2012, Salgado & Kaplitt 2015). These inputs converge and are integrated by MSNs, which

project to subcortical areas such as the globus pallidus and substantia nigra (Groenewegen et al 1999, Heimer et al 1991, Mogenson et al 1983, Salgado & Kaplitt 2015, Williams et al 1977, Zahm & Heimer 1993). Together, and along with other brain regions, afferent projections to and efferent projections from the AcbC are part of the reward circuitry of the brain (Knowland & Lim 2018, Nestler 2015, Russo & Nestler 2013). These circuits ultimately influence reward and motivated behaviors and disorders that have shown differential expression in incidence and phenotype across the estrous cycle in rodents and the menstrual cycle in humans (Alonso-Caraballo & Ferrario 2019, Banis & Lorist 2017, Becker 2016, Becker et al 2012, Ma et al 2020, Thomas & Czoty 2019, Tonn Eisinger et al 2018a, Yoest et al 2014). Important to ovarian hormone influence, recent findings have shown that, in rodents, AcbC MSNs exclusively express membrane-associated estrogen receptor (mER) α, β, and GPER-1 (Almey et al 2012) as well as progesterone receptors, albeit in the domestic hen (Sterling et al 1987). Although evidence for progesterone receptor expression and action is scant, several studies have put forward possible mechanisms for estradiol action in the AcbC. These mechanisms involve the coupling of mERα and mERβ with metabotropic glutamate receptors (mGluRs) through the concerted action of caveolin proteins and palmitoylation processes to induce short- and long-term changes to MSN structure and function (Grove-Strawser et al 2010, Martinez et al 2016, Martinez et al 2014, Meitzen et al 2013, Mermelstein et al 1996, Miller et al 2020, Peterson et al 2015, Tonn Eisinger et al 2018a, Tonn Eisinger et al 2018b). In addition to ovarian hormone action on AcbC MSNs, dopaminergic and glutamatergic inputs to the AcbC also exhibit responsiveness to biological sex, estrous cycle, and ovarian hormone influence. Here, I will detail dopaminergic and glutamatergic responsiveness to these natural variables given that these neurotransmitter systems comprise the main inputs that determine AcbC function.

Dopaminergic Action

Reports dating to the 1970s have documented sex differences in AcbC dopamine systems. This led to investigations of estrous cycle and ovarian hormone effects on this neurotransmitter system. The AcbC receives dense dopaminergic projections from the VTA (Han et al 2017). In the context of the estrous cycle, VTA dopaminergic neurons exhibit maximal firing and increases in burst firing during the estrus phase of the estrous cycle, when hormone levels decrease but hormone effects remain (Calipari et al 2017). High estradiol, sourced either through the natural ovarian cycle or through acute artificial administration, also induces an increase in K^+-induced dopamine release and an attenuation of dopamine reuptake (Thompson & Moss 1994). In addition to changes observed in dopamine availability during select estrous cycle phases and upon estradiol administration, dopamine receptor expression is also sensitive to ovarian hormones. MSNs in the AcbC differentially express D1 and D2 dopamine receptors (Lu et al 1998). D2 dopamine receptors have been implicated in mediating estradiol action in the AcbC (Chavez et al 2010, Le Saux et al 2006, Silverman & Koenig 2007), even though D1 receptors may also be involved. Indeed, estradiol administration reverses the reduction in D2 dopamine receptor expression caused by ovariectomy in the rat (Le Saux et al 2006). Behaviorally, estradiol increases amphetamine-induced conditioned place preference while decreasing RGS9-2 activity (Silverman & Koenig 2007), a protein involved in D2 receptor signaling in the basal ganglia (Martemyanov et al 2008).

Though most of the research on ovarian hormone effects on dopamine dynamics has focused on the actions of estradiol, there is select evidence for progesterone action in this system as well. However, most of what is known regarding progesterone action on dopamine comes from studies conducted in the CP. Indeed, progesterone along with NMDA application onto

striatal slices from proestrus female rats enhances the release of dopamine that was normally induced by NMDA administration alone (Cabrera & Navarro 1996). Progesterone also has a biphasic effect on striatal dopamine release in ovariectomized estradiol-primed rats. Progesterone administration 2 to 12 hours before sacrifice increased spontaneous- and amphetamine-induced dopamine release, while progesterone administration 24 hours before sacrifice inhibited both the spontaneous- and amphetamine release of dopamine (Dluzen & Ramirez 1984). In addition, amphetamine plus a pulsatile administration of BSA-conjugated progesterone produced maximal levels of dopamine release compared to continuous BSA-conjugated progesterone application or vehicle controls (Dluzen & Ramirez 1989a, Dluzen & Ramirez 1989b). Thus, supporting the notion that there is, indeed, a close interaction between progesterone and the dopaminergic neurotransmitter system, in addition to the role of estradiol.

In a pathological context, biological sex and ovarian hormone fluctuations across the estrous and menstrual cycles modulate AcbC-mediated behaviors related to drugs of abuse by increasing dopamine levels in the nucleus accumbens (Becker 2009, Becker 2016). Female humans and rats escalate faster to addictive phenotypes, work harder to attain a drug reward, and experience more relapse incidents than males (Carpenter et al 2006, Hernandez-Avila et al 2004, Lejuez et al 2007, Lynch et al 2001, McHugh et al 2018). In women, the subjective effects of drugs of abuse are enhanced during the follicular phase of the menstrual cycle when estradiol levels gradually begin to rise and eventually reach peak levels while progesterone levels are low (Justice & de Wit 1999). Further, estradiol administration during the follicular phase enhances the subjective effects of amphetamine (Justice & de Wit 2000). In contrast, progesterone administration during the follicular phase attenuates the subjective response to cocaine and women in the luteal phase of the menstrual cycle, when progesterone levels are high and

estradiol levels are moderate, experience a reduced desire to smoke cocaine (Evans & Foltin 2006, Sofuoglu et al 2002). Similarly, female rats acquire cocaine self-administration at a faster rate than do males and treatment with estradiol enhances cocaine self-administration (Davis et al 2008, Hu & Becker 2008, Lynch 2006, Lynch et al 2001, Lynch & Taylor 2005). Rats in the estrus phase of the estrous cycle prefer higher doses of cocaine when presented with a low vs. high dose choice paradigm (Lynch 2006, Lynch et al 2000). In a similar pattern to women, acute doses of progesterone decrease cocaine induced place preference in female rats (Russo et al 2003). Thus, estradiol and progesterone modulate the rewarding value attributed to drugs of abuse by modulating the dopaminergic neurotransmitter system. Although there is strong evidence for ovarian hormone modulation of dopaminergic action in the AcbC, dopamine alone does not drive MSN output activity. As mentioned before, the AcbC also receives glutamatergic projections from cortex, amygdala, and hippocampus (Berridge & Kringelbach 2008, de Jong et al 2019, Park et al 2019, Salamone 1994, Salamone & Correa 2012, Salgado & Kaplitt 2015). These projections form the basis for glutamatergic excitatory synaptic transmission onto the AcbC, which have also shown sensitivity to biological sex and ovarian hormones.

Glutamatergic Action

Sex differences and ovarian hormone sensitivity have also been discovered in glutamatergic excitatory synaptic transmission onto the AcbC. Morphologically, AcbC MSNs express densely packed dendritic spines upon which glutamatergic afferents from cortex, amygdala, and hippocampus form excitatory synapses (Groenewegen et al 1999, Kauer & Malenka 2007, Sesack et al 2003). Electron microscopy and immunocytochemistry anatomical studies revealed that there are sex differences in excitatory synapse markers in AcbC MSNs between gonad-intact females compared to gonad-intact males (Forlano & Woolley 2010,

Wissman et al 2012, Wissman et al 2011). Indeed, proestrus female rats have greater dendritic spine density as well as increased numbers of large head spines as opposed to males (Forlano & Woolley 2010). This sex difference in dendritic spine density and spine type exists along a rostro-caudal gradient. There is greater spine density in caudal AcbC, while large head spines are more prevalent in rostral AcbC (Wissman et al 2012). Research conducted in humans has also revealed that there is a sex difference in AcbC dendritic spine density favoring women (Sazdanovic M. et al 2013). Sex differences in dendritic spine density have been shown to be modulated by estradiol in brain regions other than the AcbC (Garcia-Segura et al 1994, Gould et al 1990, Meisel & Luttrell 1990, Olmos et al 1989, Woolley et al 1990, Woolley & McEwen 1992). In the AcbC, relatively recent evidence has shown that estradiol is also implicated in regulating dendritic spine density. Indeed, A 2-day subcutaneous exposure to exogenous estradiol to ovariectomized female rats and hamsters reduces dendritic spine density in this brain region (Peterson et al 2016, Peterson et al 2015, Staffend et al 2011). In rats, this reduction in spine density is dependent of estradiol activation of ERα and mGluR5 coupling as well as by mGluR-mediated endocannabinoid release and activation of CB1 receptors (Peterson et al 2016, Peterson et al 2015).

Parallel to the findings in dendritic spine density, functional assessment of glutamatergic excitatory transmission onto AcbC MSNs employing electrophysiological methods have also revealed sex- and ovarian hormone-dependent differences. At baseline levels, select glutamatergic excitatory synaptic transmission properties measured via postsynaptic currents (mEPSCs) are different between gonad-intact adult females and males. Interestingly, sex differences in mEPSC frequency are also present in pre-pubertal rats and are likely organized by sex steroid hormone production early in life. A study by Cao and colleagues revealed that

mEPSC frequency is also increased in pre-pubertal female rats compared to pre-pubertal males (Cao et al 2016). Additionally, a neonatal exposure of estradiol in both pre-pubertal females and males, or a neonatal exposure of testosterone in pre-pubertal females only, abolished the increase in mEPSC frequency observed in pre-pubertal females (Cao et al 2016), indicating that estradiol and/or testosterone exposure during the neonatal critical window of development masculinizes/defeminizes a female-specific phenotype in excitatory synaptic transmission. Although these studies have mainly focused on the prolonged actions of estradiol, recent evidence suggests that estradiol also modulates glutamatergic synapse transmission markers on a more rapid time scale as well. Indeed, bath estradiol application to acute brain slices containing the AcbC rapidly decreases mEPSC frequency while increasing mEPSC amplitude in this brain region (Krentzel et al 2019), supporting the notion that rapid estradiol action in the AcbC is largely coordinated by membrane-associated estrogen receptors.

In the context of drugs of abuse, structural and functional modifications in glutamatergic excitatory synapses have also been observed in the AcbC (Martinez et al 2016, Martinez et al 2014, Peterson et al 2016, Wissman et al 2011, Wolf 2010, Wright & Dong 2020). In male rats, an assortment of drug treatments promote increases in dendritic spine density and in the number of branched spines in distal MSN dendrites (Brown & Kolb 2001, Li et al 2003, Robinson & Kolb 1997, Robinson & Kolb 1999a, Robinson & Kolb 1999b, Robinson & Kolb 2004). Similarly, in female rats, cocaine administration further increases dendritic spine density and enhances the sex difference in mEPSC frequency onto AcbC MSNs (Wissman et al 2011). Behaviorally, cocaine administration to female rats induces robust locomotor activity responses that correlate with cocaine-induced increases in MSN dendritic spine density (Wissman et al 2011). Potentiation of cocaine-induced increases in locomotor activity and dendritic spine

density are coordinated by estradiol action on mGluR5 and the activation of the endocannabinoid system in female rat AcbC (Peterson et al 2016, Peterson et al 2015).

Collectively, these findings propose a model in which sex- and estrous cycle-dependent differences on AcbC function could potentially arise from the combined actions of glutamatergic and dopaminergic inputs onto AcbC MSNs, along with hormonal context. Consistent with this model are the lack of sex differences in MSN intrinsic excitability properties in pre-pubertal female rats (Cao et al 2016) and the localization of large head spines adjacent to dopaminergic terminals in adult female but not male rats (Forlano & Woolley 2010). However, before dopaminergic and glutamatergic inputs onto AcbC MNSs can be identified as the sole sources underlying sex- and estrous-cycle dependent differences in AcbC-mediated behaviors and disorders, an exhaustive investigation of excitatory synapse and intrinsic excitability properties of AcbC MSNs needs to be conducted across the estrous cycle and between adult females and males. Thus, the work presented in this dissertation offers this exhaustive investigation and provides insight into the underlying neuroendocrine mechanisms of sex- and estrous cycle-dependent differences in AcbC function.

Conclusion

The introduction presented here illustrates estrous/menstrual cycle and hormone dependent changes in AcbC structure and function regarding dopaminergic and glutamatergic neurotransmitter systems. However, how hormonal fluctuations across the estrous cycle combined with glutamatergic and dopaminergic transmission are integrated by AcbC MSNs to alter their input and output properties is poorly understood. This represents a critical knowledge gap given the documented differences in AcbC-mediated behaviors and disorders across the estrous cycle in rodents and the menstrual cycle in humans (Alonso-Caraballo & Ferrario 2019,

Banis & Lorist 2017, Becker 2016, Knowland & Lim 2018, Ma et al 2020, Micevych et al 2017, Nicolas et al 2019, Thomas & Czoty 2019, Tonn Eisinger et al 2018a, Yoest et al 2018). Thus, the purpose of this dissertation is to elucidate estrous cycle and ovarian hormone effects on AcbC MSN excitatory synapse and intrinsic excitability properties. To answer this overarching question, AcbC MSN excitatory synapse and intrinsic excitability properties were assessed employing whole-cell patch clamp recordings, which serve as a powerful tool to evaluate the interplay between hormonal context and the functional input/output of AcbC MSNs.

An outline of the topics explored by each chapter in this dissertation is as follows:

- Chapter 2 investigates the hypothesis that the estrous cycle modulates and induces sex differences in AcbC MSN excitatory synapse and intrinsic excitability properties.
- Chapter 3 examines the relationship between circulating levels of estradiol and progesterone with AcbC MSN excitatory synapse and intrinsic excitability properties.
- Chapter 4 explores the causal role of estradiol in regulating AcbC MSN membrane excitability properties.
- Chapter 5 offers a conclusion and places the research framework followed by the work in this dissertation into the general conversation of including sex as a biological variable (SABV) in basic neuroscience research.

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CHAPTER 2

Estrous cycle-induced sex differences in medium spiny neuron excitatory synaptic transmission and intrinsic excitability in adult rat nucleus accumbens core

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Abstract

Naturally occurring hormone cycles in adult female humans and rodents create a dynamic neuroendocrine environment. These cycles include the menstrual cycle in humans, and its counterpart in rodents, the estrous cycle. These hormone fluctuations induce sex differences in the phenotypes of many behaviors, including those related to motivation, and associated disorders such as depression and addiction. This suggests that the neural substrate instrumental for these behaviors, including the nucleus accumbens core (AcbC), likewise differs between estrous cycle phases. It is unknown if the electrophysiological properties of AcbC output neurons, medium spiny neurons (MSNs), change between estrous cycle phases. This is a critical knowledge gap given that MSN electrophysiological properties are instrumental for determining AcbC output to efferent targets. Here we test whether the intrinsic electrophysiological properties of adult rat AcbC MSNs differs across female estrous cycle phases and to males. We recorded MSNs using whole cell patch-clamp technique in two experiments: the first using gonad-intact adult males and females in differing phases of the estrous cycle, and the second using gonadectomized males and females wherein estrous cycle was eliminated. MSN intrinsic electrophysiological and excitatory synaptic input properties robustly changed between female estrous cycle phases and males. Sex differences in MSN electrophysiology disappeared when the estrous cycle was eliminated. These novel findings indicate that AcbC MSN electrophysiological properties change across the estrous cycle, providing a new framework for understanding how biological sex and hormone cyclicity regulate motivated behaviors and other AcbC functions and disorders.

Introduction

Naturally occurring hormone cycles in adult female human and rodents create a dynamic neuroendocrine environment which can potentially influence neuron structure and function (Breedlove & Arnold 1981, Cahill 2014, Gorski 1985, McCarthy 2008, Micevych et al 2017, Woolley 1998, Woolley et al 1990). These cycles include the menstrual cycle in humans, and its counterpart in rodents, the estrous cycle (Hubscher et al 2005). Similar to the menstrual cycle, the estrous cycle can be divided into phases which feature differing concentrations of gonadal hormones, 17β-estradiol (estradiol) and progesterone. In the diestrus phase, circulating plasma levels of estradiol and progesterone are low. In the proestrus phase, first progesterone and then estradiol levels rapidly peak. Finally, in the estrus phase, estradiol and progesterone levels fall but hormone effects remain. It is during this phase that follicular maturation in the ovaries induce ovulation and heightened sexual receptivity. Though these cycles induce large changes in sex steroid concentrations in neural tissue, including in the striatum (Morissette et al 1992), the effects of these cycles on neuron electrophysiology are unexplored outside of a few brain regions, including the hippocampus and amygdala (Blume et al 2017, Calizo & Flanagan-Cato 2000, Cooke & Woolley 2005, Hao et al 2006, Okamoto et al 2003, Woolley 1998, Woolley & McEwen 1993). This lack of knowledge reflects a general neglect of females in basic neuroscience research (Beery & Zucker 2011, Shansky & Woolley 2016, Will et al 2017). This is unfortunate given that the robust behavioral changes in females in different estrous cycle phases must manifest in some manner in the neural substrate.

Once class of behaviors sensitive to differing estrous cycle phases include those related to reward, motivation, and associated disorders such as depression and addiction (Baran et al 2010, Baran et al 2009, Becker 1999, Becker & Hu 2008, Becker et al 2001, Becker et al 2012, Jackson

et al 2006, Lebron-Milad & Milad 2012, Milad et al 2009, Walf & Frye 2006). Investigations into the neural substrate instrumental for these behaviors and disorders have targeted striatal brain regions, including the nucleus accumbens core (AcbC). The AcbC is a crucial nexus region linking the limbic and premotor systems (Salgado & Kaplitt 2015) and, among other tasks, helps regulate reproductive reward and sexual motivation (Tonn Eisinger et al 2018). Robust sex differences and hormone sensitivity in AcbC function have been documented, primarily in dopaminergic transmission and related behaviors and disorders (Becker 1999, Becker & Hu 2008, Czoty et al 2009, Lebron-Milad & Milad 2012, Salgado & Kaplitt 2015, Yoest et al 2014). The predominant neuron type in the AcbC is the medium spiny neuron (MSN), also called the spiny projection neuron, the region's major output neuron. AcbC MSNs integrate glutamatergic, dopaminergic, and other inputs to directly regulate motivated behaviors and AcbC-related disorders. In addition to dopaminergic action, more recently sex differences and hormone-sensitivity have also been discovered in excitatory glutamatergic synapse number, markers, and activity onto MSNs (Bonansco et al 2018, Cao et al 2016, Forlano & Woolley 2010, Peterson et al 2015, Staffend et al 2011, Willett et al 2016, Wissman et al 2012, Wissman et al 2011). In females, excitatory synapse number and function as measured via miniature excitatory postsynaptic current (mEPSC) analysis is increased compared to males (Cao et al 2016, Wissman et al 2011). However, it is unknown if the electrophysiological properties of AcbC MSNs change between estrous cycles phases. This is a critical knowledge gap given that MSN electrophysiological properties are critical for determining what information is communicated to AcbC efferent targets.

Here we address this question by testing the hypothesis that the excitatory synaptic input and intrinsic electrophysiological properties of adult rat AcbC MSNs differ across female estrous

cycle phases and to males. To accomplish this, we employed whole cell patch-clamp technique to record MSNs in acute brain slices of the AcbC in two different experiments. In the first experiment, we recorded MSNs from gonad-intact males, and females in the diestrus, proestrus, and estrus phases of the estrous cycle. We discovered robust differences in MSN electrophysiological properties across the estrous cycle and between females and males. MSN excitatory synaptic input as measured via mEPSC properties was generally elevated in females in proestrus and estrus phases compared to females in diestrus phase and males. In contrast, intrinsic neuronal excitability was decreased in females in proestrus and estrus phases compared to females in diestrus phase and males. In the second experiment, we tested whether the presence of the estrous cycle was necessary for differences in MSN electrical properties between female and males. Thus, we recorded MSNs from gonadectomized males and females in which the estrous cycle was eliminated. Sex differences in MSN electrophysiology disappeared when the estrous cycle was eliminated. These findings indicate that the primary output neurons of the AcbC, the MSNs, are sensitive to natural hormone cycles in adult females, providing a new framework for understanding how changes in cellular electrophysiology regulate hormone cycle influences on motivated behaviors and other AcbC functions and disorders.

Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University or Charles River. For experiments assessing estrous cycle, female and male Sprague Dawley CD IGS rats were born from timed-pregnant females purchased from Charles River Laboratories. Rats were housed with their littermates and dam until weaning. After weaning at postnatal day (P) 21, males (n=7) were grouped housed and

females were group housed until P60 to facilitate assessment of the following phases of the estrous cycle: diestrus (n=11), proestrus (n=8), and estrus (n=7). Age at recording ranged from post-natal day (P) P70 to P85 and was matched between groups (Mean ± SEM: diestrus: P77 ± 1; proestrus: P79 ± 1; estrus: P78 ± 2; male: P78 ± 2). All animals were housed in a temperature- and light-controlled room (23 °C, 40% humidity, 12:12-hour light/dark cycle with lights turning on and off at 7 am and 7 pm, respectively) at the Biological Resource Facility of North Carolina State University. All cages were washed with polysulfone Bisphenol A [BPA] free and were filled with bedding manufactured from virgin hardwood chips (Beta Chip, NEPCO, Warrengsburg, NY) to avoid endocrine disruptors present in corncob bedding (Mani et al 2005, Markaverich et al 2002, Villalon Landeros et al 2012). Soy protein-free rodent chow (2020X, Teklad, Madison, WI USA) and glass-bottle provided water were available *ad libitum.* For experiments employing gonadectomized rats, female (n=13) and male (n=21) Sprague-Dawley CD IGS rats were purchased from Charles River, and gonadectomized at P60. Age at recording ranged from P70 to P80 and was matched between sexes (Mean ± SEM: Female: P73 ± 1; Male: P73 ± 1). Rats were housed at the Biological Resource Facility at North Carolina State University as described above.

Estrous Cycle Assessment

Estrous cycle assessment was performed using a wet mount preparation as previously described (Hubscher et al 2005). Briefly, females (P60 or older) were swabbed using potassium phosphate buffer solution (KPBS) ~10:00 am. Slides were visualized under a microscope to determine estrous cycle stage according to cell morphology as previously described (Westwood 2008). Estrous cycle stage was confirmed via assessment of plasma concentrations of progesterone, 17β-estradiol, and testosterone (Table 2.1). At sacrifice (~10:30 am), trunk blood

was collected from each subject and centrifugated within 30 minutes. Harvested plasma was stored at -20°C until assessment at the Ligand Assay and Analysis Core at the University of Virginia using commercially available ELISA kits manufactured by Calbiotech (estradiol) or IBL (progesterone, testosterone). All protocols were validated based on the recommendations of the Endocrine Society (Wierman et al 2014). Samples were run in duplicates. Intra and inter-assay percent coefficient of variations were: estradiol: 8.3, 9.9, progesterone: 5.6, 10.2, testosterone: 5.4, 7.8, respectively. The minimum detectable plasma estradiol, progesterone, and testosterone concentrations were 3 pg/mL, 0.15 ng/mL, 10 ng/dL, respectively. The maximum detectable plasma concentrations were 300 pg/mL, 40 ng/mL, 1,600 ng/dL, respectively. Plasma estradiol levels differed across estrous cycle phase in females, as expected (Butcher et al 1974). Estradiol was significantly elevated in proestrus compared to diestrus and estrus phases (Table 2.1). Progesterone levels did not significantly differ across phases, indicating that animals in the proestrus phase were used before manifestation of peak progesterone levels. Overall, plasma hormone levels matched estrous cycle phase identification from vaginal cytology assessment, confirming the validity of this method for estrous cycle phase identification.

Table 2.1. Plasma hormone concentrations in gonad intact adult rats.

Hormone	Diestrus	Proestrus	Estrus	Males	Statistics (F/KW, P)
17β-estradiol (pg/mL)	5.345 ± 1.856 (11)[a]	16.09 ± 2.469 (8)[b]	2.657 ± 1.021 (7)[a]	---	12.05, 0.0003*
Progesterone (ng/mL)	27.27 ± 3.144 (11)	23.17 ± 5.092 (8)	27.61 ± 5.317 (7)	---	0.31, 0.74*
Testosterone (ng/mL)	---	---	---	8.144 ± 1.096 (6)	---

Notes: Values are mean ± SEM. Numbers in parentheses indicate animal sample size. Acronyms: Not measured, ---. Superscript letters indicate statistically significant differences across groups. * denotes absence of normality.

Acute Brain Slice Preparation

Brain slices for electrophysiological recordings were prepared as previously described (Dorris et al 2014). Rats were deeply anesthetized with isoflurane gas and euthanized by decapitation. The brain was then dissected rapidly into ice-cold, oxygenated sucrose artificial cerebrospinal fluid (s-ACSF) containing (in mM): 75 sucrose, 1.25 NaH_2PO_4, 3 $MgCl_2$, 0.5 $CaCl_2$, 2.4 Na pyruvate, 1.3 ascorbic acid from Sigma-Aldrich, St. Louis, MO, and 75 NaCl, 25 $NaHCO_3$, 15 dextrose, 2 KCl from Fisher, Pittsburg, PA. The osmolarity of the s-ACSF was 295-305 mOsm, and the pH was between 7.2-7.4. Coronal brain slices (300 μm) were prepared using a vibratome and then incubated in regular ACSF containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$ (295-305 mOsm, pH 7.2-7.4) for 30 minutes at 30-35ºC, and then at least 30 minutes at room temperature (22-23ºC). Slices were stored submerged in room temperature, oxygenated ACSF for up to 5 hours after sectioning in a large volume bath holder.

Electrophysiological recordings

Slices rested for at least 1 hour after sectioning. They were then placed in a Zeiss Axioscope equipped with IR-DIC optics, a Dage IR-1000 video camera, and 10X and 40X lenses with optical zoom, and superfused with oxygenated ACSF heated to ~22ºC. Whole-cell patch-clamp recordings were used to record the electrical properties of MSNs in the AcbC (Estrous cycle experiments: Figure 2.1A; Gonadectomized experiments: Figure 2.1B). Glass electrodes (6-23 MΩ) contained (in mM): 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 $MgCl_2$, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine from Sigma-Aldrich and 10 HEPES from Fisher, 285 mOsm, pH 7.2-7.4. Signals were amplified, filtered (2 kHz), and digitized (10 kHz) with a MultiClamp 700B amplifier attached to a Digidata 1550 system and a personal computer using pClamp 10

software. Membrane potentials were corrected for a calculated liquid junction potential of -13.5 mV. Using previously described procedures (Dorris et al 2015), recordings were first made in current clamp to assess neuronal electrophysiological properties. MSNs were identified by their medium-sized somas, the presence of a slow ramping subthreshold depolarization in response to low-magnitude positive current injections, an action potential amplitude >20 mV, a hyperpolarized resting potential more negative than -65 mV, inward rectification, and prominent spike afterhyperpolarization (Belleau & Warren 2000, O'Donnell & Grace 1993).

In a subset of recordings, oxygenated ACSF containing both the $GABA_A$ receptor antagonist Picrotoxin (PTX, 150 μM; Fisher) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 μm, Abcam Biochemicals) was applied to the bath to abolish inhibitory post-synaptic current events and action potentials, respectively. Once depolarizing current injection no longer generated an action potential, MSNs were voltage clamped at -70 mV and miniature excitatory post-synaptic currents (mEPSCs) were recorded for at least five minutes. These settings enable recordings from almost exclusively AMPA glutamate receptors (Nowak et al 1984). To confirm that mEPSCs were generated from AMPA glutamate receptors in the current experiment, we exposed two MSNs to oxygenated ACSF containing the AMPA receptor antagonist 6,7-Dinitroquinoxaline-2,3-dione disodium salt (DNQX, 25 μM; Tocris) in addition to PTX and TTX. DNQX exposure eliminated mEPSCs, lowering mEPSC frequency to 0.01% of pre-DNQX exposed values. In all experiments, input/series resistance was monitored for changes and cells were excluded if resistance changed more than 25%.

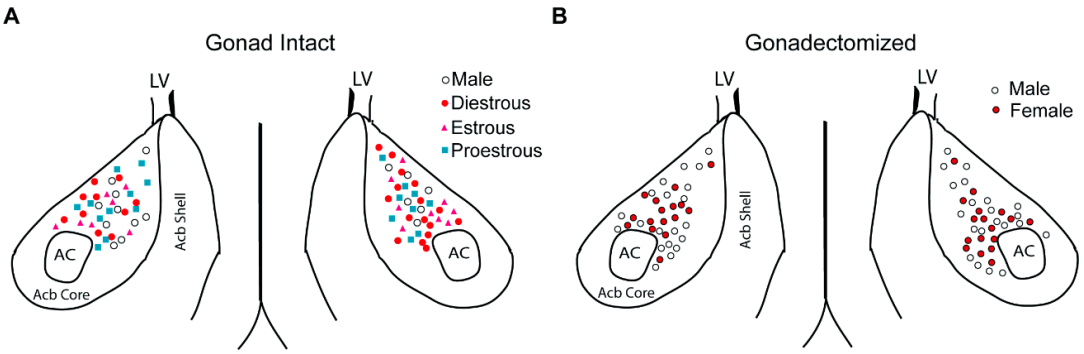

Figure 2.1. Location of whole cell patch-clamped medium spiny neurons (MSNs) in rat nucleus accumbens core (AcbC). A) Gonad-intact females in differing estrous cycle phases and males. B) Gonadectomized females and males. Acronyms: AC, anterior commissure; LV, lateral ventricle; Acb, nucleus accumbens.

Data Recording and Analysis

Intrinsic electrophysiological properties and action potential characteristics were analyzed using pClamp 10. After break-in, the resting membrane potential was first allowed to stabilize ~1-2 minutes, as in (Mu et al 2010). At least three series of depolarizing and hyperpolarizing current injections were applied to elicit basic neurophysiological properties. Most properties measured followed the definitions of (Cao et al 2016, Dorris et al 2015, Willett et al 2018, Willett et al 2016), which were based upon those of Perkel and colleagues (Farries et al 2005, Farries & Perkel 2000, Farries & Perkel 2002, Meitzen et al 2009). For each neuron, measurements were made of at least three action potentials generated from minimal current injections. These measurements were then averaged to generate the reported action potential measurement for that neuron. For action potential measurements, only the first generated action potential was used unless more action potentials were required to meet the standard three action potentials per neuron. Action potential threshold was defined as the first point of sustained positive acceleration of voltage ($\delta^2V/\delta t^2$) that was also more than three times the standard deviation of membrane noise before the detected threshold (Baufreton et al 2005). The delay to first action potential is the average time in milliseconds of the time from the initial deflection from the current step function to the action potential threshold of the first spike. Action potential

width at half peak is the width of the action potential half-way between action potential peak and threshold in milliseconds (ms). The action potential amplitude is the change in millivolts between action potential threshold and peak. After-hyperpolarization peak amplitude is the difference in millivolts between action potential threshold and the lowest point after peak measured from threshold value after peak to lowest point. After-hyperpolarization time to peak amplitude is the time measured in milliseconds of the time from initial deflection from the current step function to action potential threshold of the first spike. Rheobase, measured in nanoamps (nA) is the lowest amplitude of injected positive current needed to produce an initial action potential. The slope of the linear range of the evoked action potential firing rate to positive injected current curve (FI slope) was calculated from the first current stimulus which evoked an action potential to the first current stimulus that generated an evoked firing rate that persisted for at least two consecutive current stimuli. Minimum firing rate was defined as the action potential rate generated in response to a 600 ms rheobase current. Maximum firing rate was defined as the action potential rate generated in response to a +0.1 nA injected current. Resting membrane potential is the average of the statistical mean value of the baseline of all traces from the negative current step files. Input resistance in the linear, non-rectified range was calculated from the steady-state membrane potential in response to −0.02 nA hyperpolarizing pulses. Rectified range input resistance and percent inward rectification was calculated as described previously, with rectified range input resistance measured using the most hyperpolarizing current injected into the MSN (Belleau & Warren 2000). Inward rectification is the input resistance of the -0.02 nA step minus the rectified range input resistance. Percent inward rectification is defined as: RRIR/IR x 100. The time constant of the membrane was calculated by fitting a single exponential curve to the membrane potential change in response to −0.02 nA hyperpolarizing pulses. Possible sex

differences in hyperpolarization-induced "sag" (i.e., I_H current) were assessed using the "sag index" (Farries et al. 2005). Briefly, sag index is defined as the difference between the minimum voltage measured during the largest hyperpolarizing current pulse and the steady-state voltage deflection of that pulse, divided by the steady-state voltage deflection. A cell with no sag would exhibit a sag index of 0, whereas a cell whose maximum voltage deflection is twice that of the steady-state deflection would exhibit a sag index of 1. Cells with considerable sag typically have an index of more than 0.1. Frequency, amplitude, and decay of mEPSCs were analyzed off-line using Mini Analysis (Synaptosoft, http://www.synaptosoft.com/MiniAnalysis/). mEPSC threshold was set at a minimum value of 5 picoamps (pA), and accurate event detection was validated by visual inspection. mEPSC frequency was defined as the number of detected mEPSC events per second (Hz). mEPSC amplitude was calculated as the difference between the averaged baseline 10 msec before initial mEPSC rise and peak mEPSC amplitude. mEPSC decay was calculated as the time required for peak mEPSC amplitude to return to the averaged baseline.

Statistics

Experiments were analyzed as appropriate using one-way ANOVAs with Newman-Keuls post-hoc tests or Kruskal-Wallis tests with Dunn's correction for multiple comparisons as well as linear regression tests. Distributions were analyzed for normality using D'Agostino & Pearson omnibus normality test (Prism). An *a priori* outlier analysis was performed and values falling more than 4 SDs from the mean were excluded from analysis. A total number of 4 cells were excluded from analysis. P values < 0.05 were considered *a priori* as significant. Data are presented as mean ± SEM.

Results

Here, we comprehensively test the hypothesis that estrous cycle influences female rat MSN electrophysiology, including both excitatory synaptic properties and intrinsic membrane properties. To accomplish this goal, we performed two separate experiments. First, we tested how the different phases of the estrous cycle with concomitant cyclical changes in hormone concentrations modulated MSN electrophysiological properties in gonad-intact rats. Second, we gonadectomized female and male rats to test if sex differences in MSN electrophysiological properties remained after abolition of the estrous cycle. In this results section, we will first address the effects of the estrous cycle in gonad-intact rats, and then follow with experiments in gonadectomized rats.

Experiment 1: Gonad-intact rats

mEPSC frequency decreases in diestrus compared to proestrus and estrus

Previous work has identified sex differences in mEPSC properties in AcbC MSNs. Most notably, mEPSC frequency was increased in prepubertal females compared to prepubertal males, and in adult females of unknown estrous phase compared to adult males (Cao et al 2016, Wissman et al 2011). Thus, it is possible that mEPSC properties may vary across the estrous cycle. To test this hypothesis, we voltage clamped MSNs to -70 mV and recorded mEPSCs while exposing MSNs to 1 μM TTX and 150 μM PTX to block sodium dependent action potentials and $GABA_A$ receptors, respectively (Figure 2.2A). We assessed mEPSC frequency, amplitude, and decay. Complete statistical information can be found in Table 2.2. mEPSC frequency was significantly elevated in the proestrus and estrus phases in females compared to diestrus phase females and males (Figure 2.2B). mEPSC amplitude was significantly decreased in estrus phase females compared to males (Figure 2.2C). mEPSC amplitude was also significantly decreased in

diestrus phase females compared to males (Figure 2.2C). mEPSC decay was significantly decreased in estrus phase females compared to all other groups, with mEPSC decay being the longest in males (Figure 2.2D). mEPSC decays can be longer or shorter because of changes in decay rate, or because of changes in mEPSC amplitude while decay rate remains constant. To differentiate between these possibilities, we plotted mEPSC decay versus mEPSC amplitude (Figure 2.2E). We then analyzed the data using linear regressions, which found significant correlations between mEPSC amplitude and mEPSC decay in diestrus and proestrus phase females and a trend in estrus phase females (Diestrus: R^2: 0.57, F: 22.22, P=0.0002; Proestrus: R^2: 0.66, F: 19.44, P=0.0013; Estrus: R^2: 0.23, F: 3.51, P<0.086; Males: R^2: 0.075, F: 0.65, P=0.44). This correlation-based analysis indicates that the differences in mEPSC decay in most female estrous cycle stages are primarily driven by changes in mEPSC amplitude, and not differences in decay rate. This lack of difference in mEPSC decay in females is in contrast to males, which exhibit uncorrelated changes in mEPSC decay and amplitude. This lack of correlation suggests that males display independent differences in both mEPSC decay and amplitude compared to females. To further probe this phenomenon, we calculated a normalized mEPSC decay tau value taken at 10-90% of mEPSC amplitude for each experimental group (KW=14.62, P=0.022; diestrus females: 1.00 ± 0.09; proestrus females: 0.93 ± 0.10; estrus females: 0.88 ± 0.08; males: 0.54 ± 0.05). Normalized mEPSC decay tau did not differ between diestrus, proestrus and estrus phase females (P>0.05 for all comparisons), but males differed from females in diestrus (P<0.01), proestrus (P<0.05), and estrus (P<0.05). This analysis supports the conclusion that females primarily show differences in mEPSC frequency across estrous cycle phases and differ from males but not each other in both mEPSC decay and amplitude. It also supports the conclusion that differences in mEPSC decay between males and

females are not solely due to differences in mEPSC amplitude. Overall, these findings show that excitatory synaptic input as assessed by mEPSC properties varies robustly across the estrous cycle.

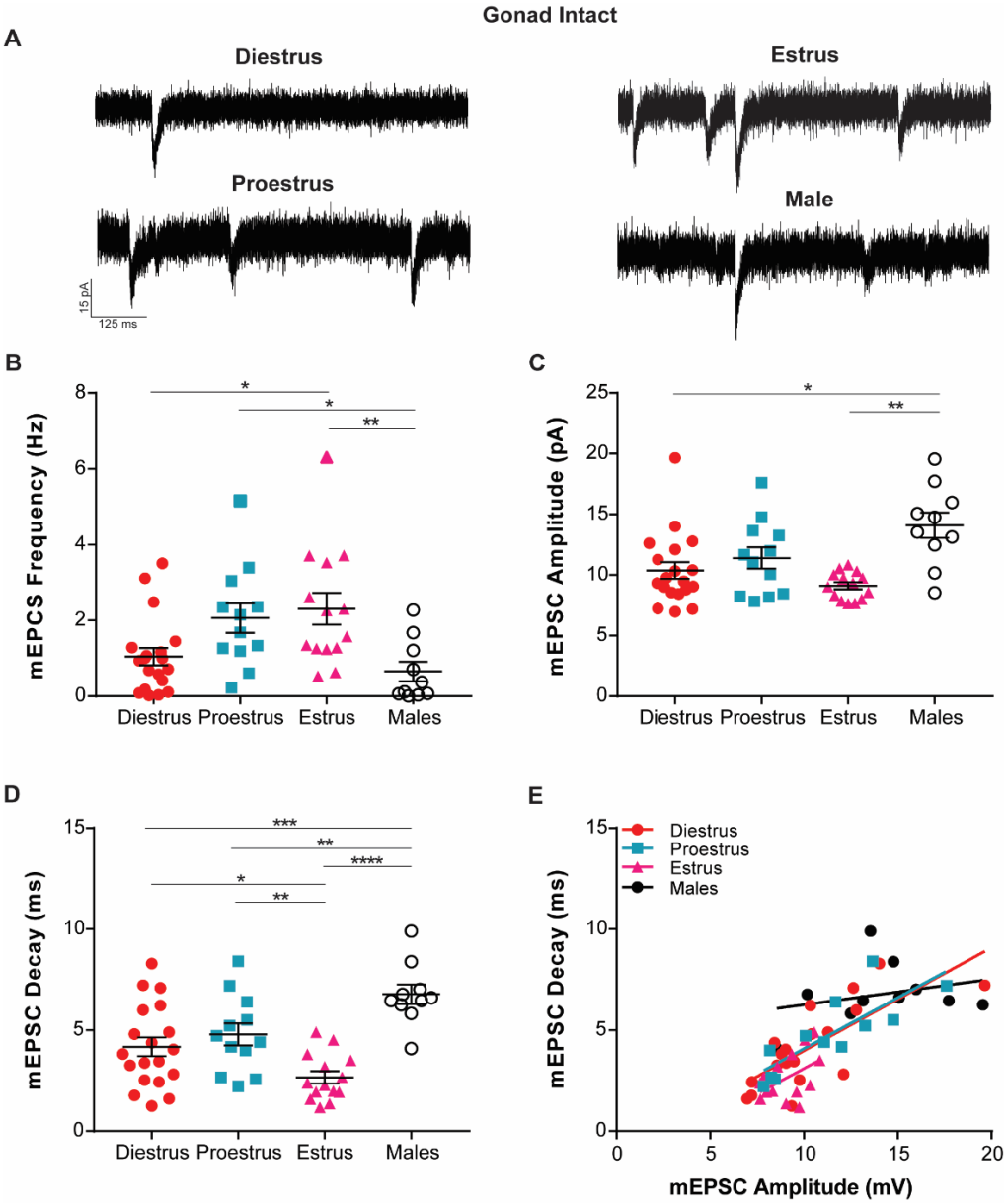

Figure 2.2. MSN mEPSC properties: gonad-intact females and males. A) Representative examples of miniature excitatory postsynaptic currents (mEPSCs) recorded in diestrus, proestrus, and estrus female and male MSNs. The following properties varied across estrous cycle phases in females and/or to males: B) mEPSC frequency, C) mEPSC amplitude, D) mEPSC decay. E) mEPSC amplitude was significantly correlated with mEPSC decay in diestrus, proestrus, and estrus females but not males. The horizontal line superimposed upon scatter plots in Figures B through D indicates the mean. The lines situated above scatter plots indicate statistical significance. Complete statistical information is in Table 2.2. Acronyms: mEPSC, miniature excitatory postsynaptic currents.

Table 2.2. mEPSC properties recorded from MSNs in gonad intact adult rat AcbC.

Property	Diestrus	Proestrus	Estrus	Male	Statistics (F/KW, P)
Frequency (Hz)	1.05 ± 0.23 (19)[a, b]	2.07 ± 0.39 (12)[a, c]	2.31 ± 0.42 (14)[c]	0.65 ± 0.25 (10)[b]	16.11, 0.001*
Amplitude (pA)	10.37 ± 0.69 (19)[a]	11.40 ± 0.88 (12)[a, b]	9.11 ± 0.30 (14)[a]	14.10 ± 1.04 (10)[b]	13.85, 0.003*
Decay (ms)	4.17 ± 0.46 (19)[a]	4.79 ± 0.55 (12)[a]	2.66 ± 0.31 (14)[b]	6.78 ± 0.48 (10)[c]	11.38, <0.0001

Notes: Values are mean ± SEM. Numbers in parentheses indicate number of neurons recorded. Shaded values indicate statistical significance. Superscript letters indicate statistically significant differences across groups. * denotes absence of normality.

Most individual action potential properties do not differ between estrous cycle phases

We also tested whether intrinsic MSN electrophysiological properties differed between estrous cycle phases, including individual action potential properties, excitability, and passive membrane properties. To accomplish this, we injected MSNs with a series of positive and negative current injections and analyzed a battery of electrophysiological attributes. These electrophysiological attributes and related statistical information are provided in Table 2.3. Regarding action potential properties (Figure 2.3A), individual action potentials recorded from MSNs in diestrus phase females trended towards a shorter delay to first action potential in comparison to proestrus and estrus phase females and to males (Figure 2.3B; Table 2.3; P=0.0545). This delay is a canonical feature of MSNs and reflects the underlying slow inactivating A-current (Nisenbaum et al 1994). Action potential width was significantly longer in diestrus and proestrus phase females compared to males, where the overall average change was less than a millisecond (Figure 2.3C). No differences were detected in action potential threshold (Figure 2.3D), action potential amplitude (Figure 2.3E), the action potential afterhyperpolarization time to peak amplitude (Figure 2.3F), or the action potential afterhyperpolarization peak amplitude (Figure 2.3G). These findings indicate that most individual action potential properties do not differ across the estrous cycle, except for a relatively minor change in action potential width.

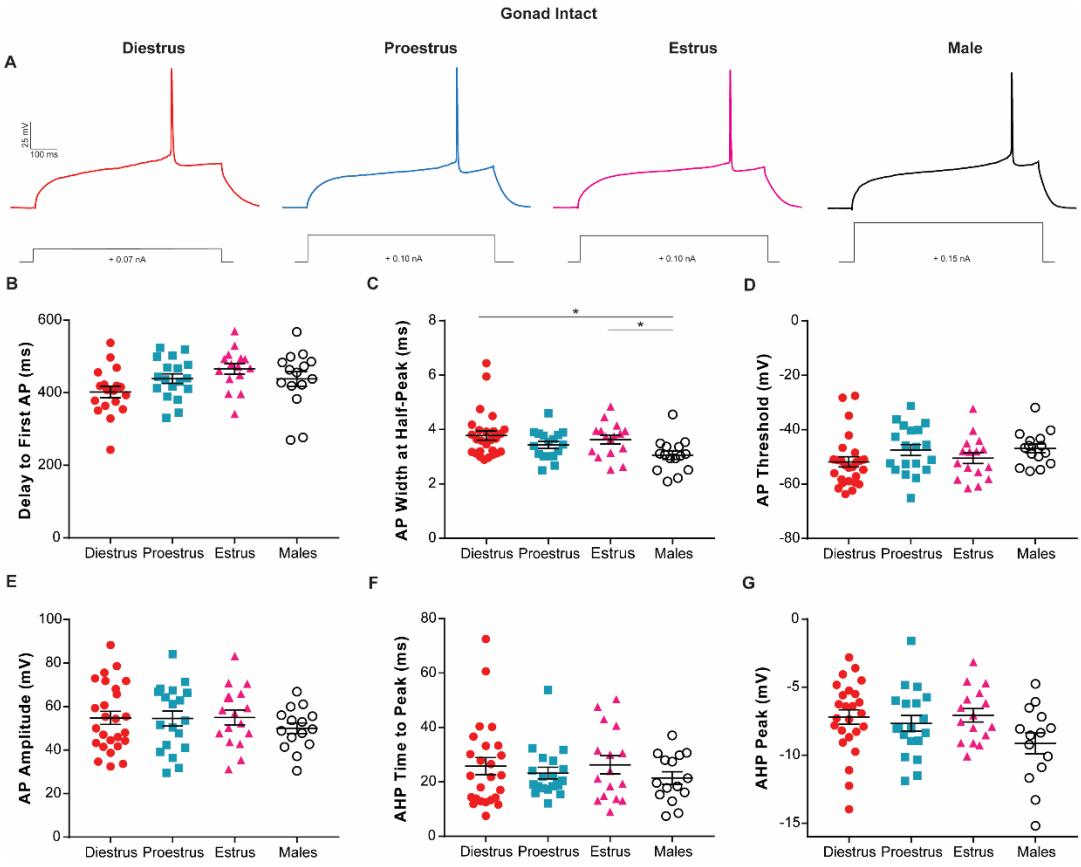

Figure 2.3. MSN individual action potential properties: gonad-intact females and males. A) Voltage responses of diestrus, proestrus, and estrus female and male MSNs to a single depolarizing current injection. The following action potential property varied across estrous cycle and/or to males but remained a statistical trend: B) Delay to first action potential. The following action potential property varied across estrous cycle and/or to males: C) action potential width measured at half peak amplitude. The following action potential properties did not vary across estrous cycle phases in females and/or to males: D) action potential threshold, E) action potential amplitude, F) action potential afterhyperpolarization time to peak amplitude, G) action potential afterhyperpolarization peak amplitude. The horizontal line superimposed upon scatter plots in Figures B through G indicates the mean. The lines situated above scatter plots indicate statistical significance. Complete statistical information is in Table 2.3. Acronyms: AP, action potential; AHP, afterhyperpolarization.

Rheobase, the amount of current required for action potential initiation, decreases in diestrus compared to proestrus and estrus

To test the hypotheses that action potential initiation and generation varied across estrous cycle phases, we injected a series of positive current injections into MSNs and analyzed the initiation and number of evoked action potentials (Figure 2.4A). Regarding action potential initiation, the minimum amount of excitatory current necessary to trigger an action potential significantly varied between estrous cycle phases (Figure 2.4B). Rheobase was drastically

reduced in MSNs recorded in diestrus phase females compared to proestrus phase females and males. Changes in rheobase are sometimes concomitant with changes in action potential threshold, resting membrane potential, and/or input resistance. Since there were no changes in action potential threshold between estrous cycle phases (Figure 2.3C), we next focused on analyzing resting membrane potential. Resting membrane potential was depolarized in MSNs recorded from diestrus phase females compared to proestrus and estrus phase females and males (Figure 2.4C). Interestingly, after action potential initiation, the frequency of action potentials evoked by depolarizing current injection did not differ by estrous cycle phase, including minimum firing rate (Figure 2.4D), maximum firing rate (Table 2.3), and the slope of the frequency of action potentials evoked by depolarizing current injection curve (FI Curve) (Figure 2.4E). Thus, MSNs in diestrus exhibit increased excitability in the sense that action potential rheobase is decreased, indicating that less depolarizing current is required for initial action potential generation.

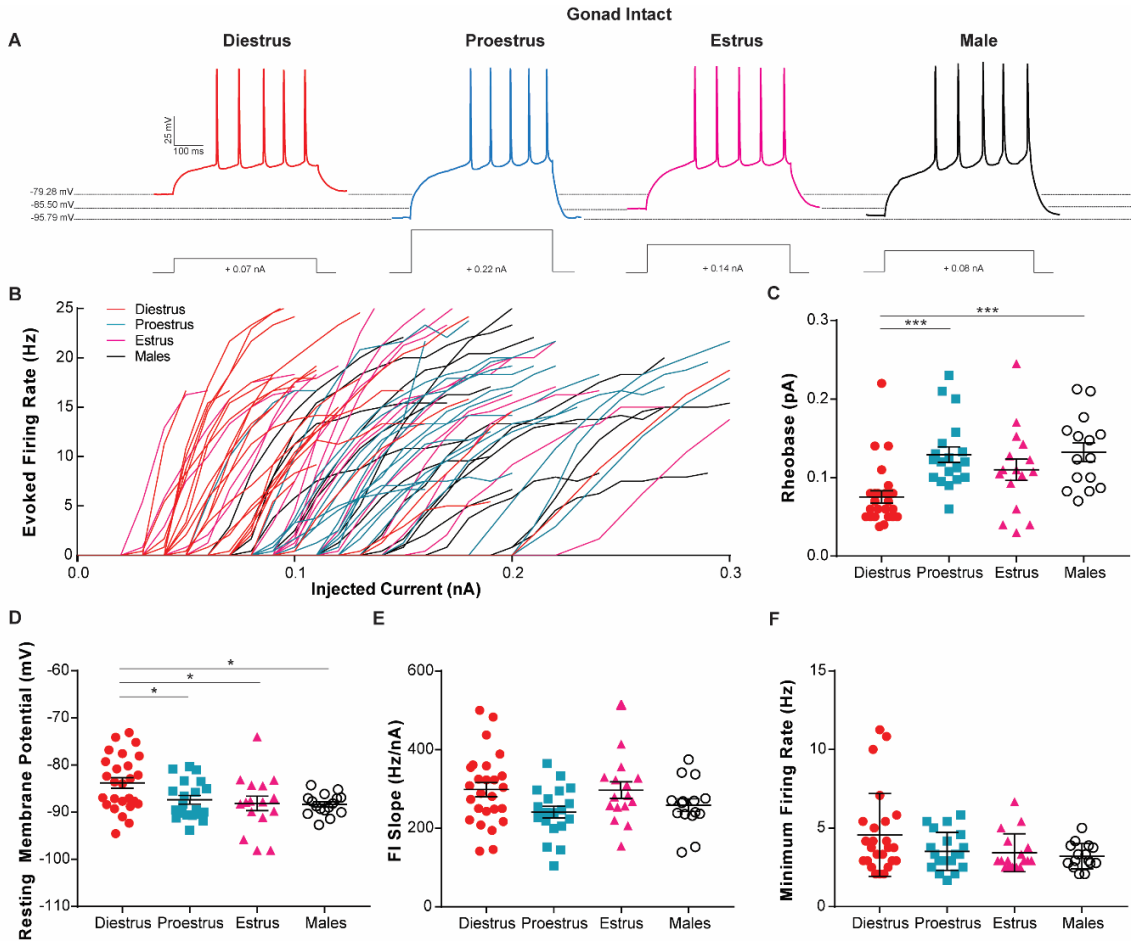

Figure 2.4. MSN action potential initiation and generation: gonad-intact females and males. A) Voltage responses of diestrus, proestrus, and estrus female and male MSNs to a series of depolarizing current injections. B) Action potential firing rates evoked by depolarizing current injections. The following properties varied across estrous cycle phase in females and/or to males: C) rheobase, D) resting membrane potential. The following properties did not vary across estrous cycle and/or to males: E) The slopes of the evoked firing rate to positive current curve (FI Slope) in individual MSNs, F) minimum evoked firing rate. The horizontal line superimposed upon scatter plots in Figures C through F indicates the mean. The lines situated above scatter plots indicate statistical significance. Complete statistical information is in Table 2.3. Acronyms: FI Slope, slope of the evoked firing rate to positive current curve.

Input resistance increases in diestrus compared to proestrus females and males

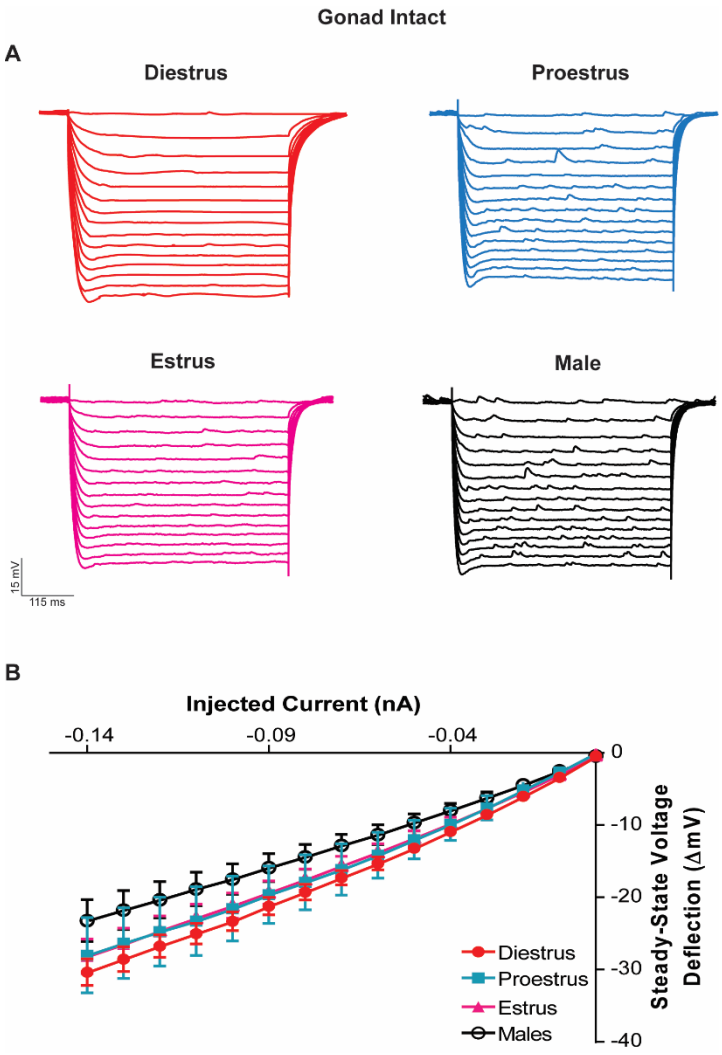

Figure 2.5. MSN passive electrophysiological properties: gonad-intact females and males. A) Voltage response of a diestrus, proestrus, and estrus female and male MSNs to a series of hyperpolarizing current injections. B) The injected current to steady stage voltage deflection curve (IV curve) varied across estrous cycle in females and/or to males.

The above-mentioned decrease in rheobase in diestrus compared to other estrous cycle phases and males may also be driven by an increase in input resistance. To investigate input resistance and other passive membrane properties, we injected a series of negative current injections in MSNs across female estrous cycle phases and males (Figure 2.5A). When we plotted the steady-state voltage deflection evoked by injected hyperpolarizing current curve (IV curve) (Figure 2.5B), MSNs recorded from females in diestrus phase showed increased voltage

deflections in response to higher magnitude hyperpolarizing current injections compared to males and females in other estrous cycle phases. We further evaluated this by measuring input resistance in both the linear and rectified range. Input resistance in the linear range was increased in MSNs recorded from diestrus phase females compared to proestrus phase females and males (Figure 2.6A). Input resistance in the rectified range was also increased in MSNs recorded from diestrus phase females compared to proestrus phase females and males (Figure 2.6B). Additionally, the time constant of the membrane was longer in MSNs recorded from diestrus phase females compared to males (Figure 2.6C). This increase in input resistance and time constant of the membrane in MSNs recorded from diestrus phase females is consistent with the previously mentioned decreased rheobase in this phase.

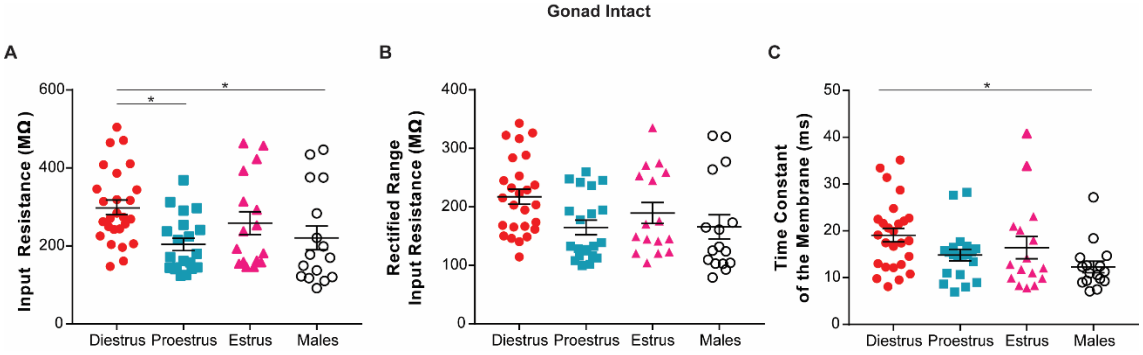

Figure 2.6. MSN input resistance and time constant of the membrane properties: gonad-intact females and males. The following properties varied across estrous cycle in females and/or to males: A) input resistance in the linear range, B) input resistance in the rectified range. The overall ANOVA for input resistance in the rectified range was significant but no differences were detected by the post-hoc test. C) time constant of the membrane. The horizontal line superimposed upon scatter plots in Figures A through C indicates the mean. The lines situated above scatter plots indicate statistical significance. Complete statistical information is in Table 2.3.

Table 2.3. Electrophysiological properties of MSNs in adult gonad intact rat AcbC.

Property	Diestrus	Proestrus	Estrus	Male	Statistics (F/KW, P)
Resting membrane potential (mV)	-83.81±1.14 (26)[a]	-87.37±0.89 (20)[b]	-88.13±1.51 (16)[b]	-88.34±0.56 (16)[b]	4.17, 0.009
Delay to first AP (ms)	402.30±15.59 (18)	438.90±13.15 (18)	465.90±14.63 (15)	438.20±20.70 (15)	2.68, 0.0545
Rheobase (nA)	0.08±0.01 (26)[a]	0.13±0.01 (19)[b, c]	0.11±0.01 (16)[c]	0.13±0.01 (15)[b, c]	23.15, <0.0001*
AP threshold (mV)	-51.81±1.86 (26)	-47.44±2.02 (19)	-50.45±1.97 (16)	-46.86±1.62 (15)	6.97, 0.07*
AP width at half peak amplitude (ms)	3.78 ± 0.17 (26)[a]	3.44±0.13 (17)[a, b]	3.63±0.16 (16)[a]	3.06 ± 0.16 (15)[b]	11.23, 0.01*
AP amplitude (mV)	54.79±3.01 (26)	54.55±3.47 (19)	54.99±3.47 (16)	50.03±2.48 (15)	0.46, 0.71
AHP peak amplitude (mV)	-7.20±0.53 (25)	-7.66±0.58 (19)	-7.06±0.49 (16)	-9.13±0.77 (14)	2.16, 0.10
AHP time to peak (ms)	25.82±3.14 (25)	23.21±2.13 (19)	26.28±3.33 (16)	21.41±2.28 (15)	0.70, 0.87*
FI Slope (Hz/nA)	298.70±17.85 (26)	241.30±14.84 (19)	297.10±21.20 (16)	258.60±16.23 (15)	2.58, 0.06
Time constant of the membrane (ms)	19.07±1.45 (26)[a]	14.83±1.23 (20)[a, b]	16.42±2.38 (16)[a, b]	12.29±1.22 (16)[b]	11.31, 0.01*
Linear range input resistance (MΩ)	299.10±18.67 (26)[a]	203.90±16.01 (20)[b]	258.60±29.36 (16)[a, b]	220.80±30.63 (16)[b]	3.92, 0.01
Rectified range input resistance (MΩ)	217.50±12.72 (26)	164.80±12.60 (20)	189.50±17.88 (16)	165.90±20.66 (16)	2.94, 0.04
Percent inward rectification (%)	74.92±3.03 (26)	81.36±1.99 (20)	77.92±4.10 (16)	78.98±3.44 (16)	1.90, 0.59*
Inward rectification (MΩ)	81.65±14.08 (26)	39.03±5.91 (20)	69.05±19.00 (16)	54.83±16.18 (16)	6.10, 0.11*
Sag Index (unitless)	0.004±0.004 (26)	0.003±0.001 (20)	0.005±0.004 (16)	0.008±0.004 (16)	1.48, 0.69*
Minimum Firing Rate (Hz)	4.57±0.53 (25)	3.52±0.27 (20)	3.43±0.29 (17)	3.21±0.21 (15)	3.54, 0.32*
Maximum Firing Rate (Hz)	17.70±1.12 (25)	18.16±1.11 (20)	21.10±1.51 (17)	17.62±1.52 (15)	4.01, 0.26*

Notes: Values are mean ± SEM. Numbers in parentheses indicate number of neurons recorded. Shaded values indicate statistical significance. Acronyms: Action potential, AP; Afterhyperpolarization, AHP; Frequency of evoked action potentials to injected depolarizing current, FI. Superscript letters indicate statistically significant differences across groups. * denotes absence of normality.

Experiment 2: Gonadectomized rats

If these changes in MSN excitatory synapse properties and intrinsic electrophysiological properties are driven by the naturally occurring estrous cycle, then elimination of the estrous cycle should likewise eliminate sex differences in MSN electrophysiology. Therefore, to test the hypothesis that the estrous cycle is necessary to induce sex differences in AcbC MSN

electrophysiological properties, we gonadectomized adult female and male rats at P60 by removing the ovaries and testes, respectively. After surgery, we waited two weeks and then recorded MSNs. All MSN electrophysiological properties and statistics can be found in Table 2.4 and Table 2.5.

The estrous cycle is necessary for sex differences in mEPSC properties

We first present excitatory synaptic input properties, given that these differed by estrous cycle in gonad-intact animals (Figure 2.2). MSNs from gonadectomized females and males were voltage clamped to -70 mV and mEPSCs were recorded during exposure to 1 µM TTX and 150 µM PTX. mEPSC frequency, amplitude and decay were analyzed to evaluate excitatory synaptic input (Table 2.4). mEPSC frequency (Figure 2.7A), mEPSC amplitude (Figure 2.7B), and mEPSC decay (Figure 2.7C) did not differ between females and males as it did in intact animals. Thus, the presence of the estrous cycle is necessary for sex differences in mEPSC properties.

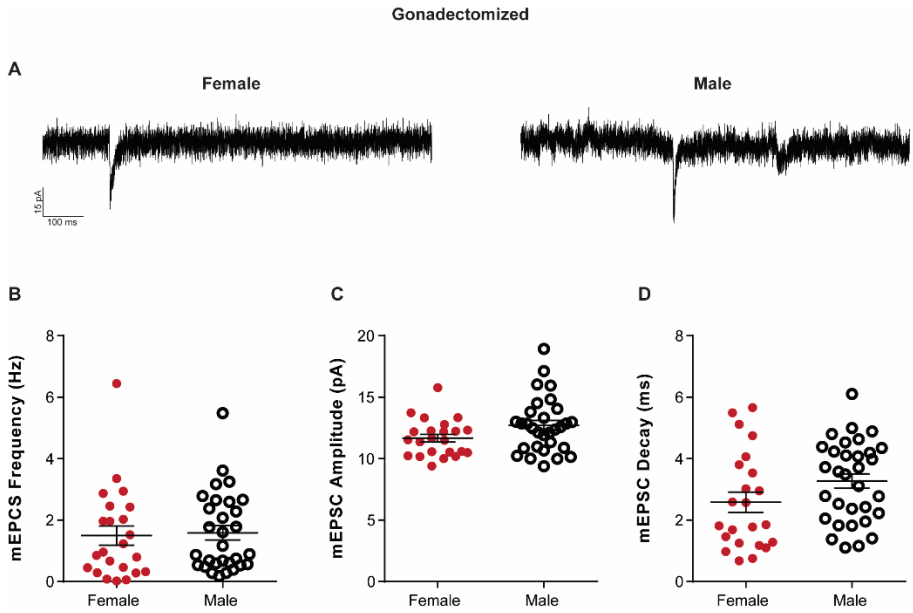

Figure 2.7. MSN mEPSC properties: gonadectomized females and males. A) Representative examples of miniature excitatory postsynaptic currents (mEPSCs) recorded in gonadectomized female and male MSNs. The following properties did not vary between gonadectomized females and males: B) mEPSC frequency, C) mEPSC amplitude, D) mEPSC decay. The horizontal line superimposed upon scatter plots in Figures B through D indicates the mean. Complete statistical information is in Table 2.4. Acronyms: mEPSC, miniature excitatory postsynaptic currents.

Table 2.4. mEPSC properties recorded from medium spiny neurons in adult gonadectomized rat nucleus accumbens core.

Property	Female	Male	Statistics (*t*/U, P)
Frequency (Hz)	1.50 ± 0.31 (23)	1.59 ± 0.23 (30)	310, 0.54*
Amplitude (pA)	11.67 ± 0.31 (23)	12.71 ± 0.41 (30)	1.91, 0.06
Decay (ms)	2.58 ± 0.33 (23)	3.27 ± 0.23 (30)	1.77, 0.08

Notes: Values are mean ± SEM. Numbers in parentheses indicate sample size. No comparisons reached statistical significance. * denotes absence of normality.

Individual action potential properties do not differ by sex in gonadectomized rats

In gonad-intact animals, no sex or estrous cycle phase differences were detected in individual action potential properties apart from a relatively small difference in action potential width (Figure 2.3C). To further corroborate that AcbC MSNs exhibit minimal differences in individual action potential properties, action potentials were evoked from MSNs from gonadectomized females and males by injecting positive current (Figure 2.8A). No differences were detected in the delay to first action potential (Figure 2.8B), action potential threshold (Figure 2.8C), and action potential width measured at half peak amplitude (Figure 2.8D). Gonadectomized male MSNs showed a small but significant increased mean action potential amplitude compared to gonadectomized female MSNs (Figure 2.8E; p=0.04). Given that the range of gonadectomized female and male action potential amplitudes were similar (Female: 10% Percentile 38.65 mV, 90% Percentile 73.2 mV; Male: 10% Percentile 40.18 mV, 90% Percentile 78.99 mV), we further assessed the distributions of action potential amplitudes (Figure 2.8F). Action potential amplitude distributions did not differ between MSNs recorded from gonadectomized females and males (p=0.0880). This lack of statistical significance between action potential amplitude distributions suggests that this is not a robust sex difference. Action potential afterhyperpolarization peak magnitude (Figure 2.8G) or time to afterhyperpolarization peak magnitude (Figure 2.8H) also did not differ between gonadectomized females and males. Overall, this analysis indicates that the MSN individual action potential properties are comparable across sex, similar to findings from gonad-intact rats.

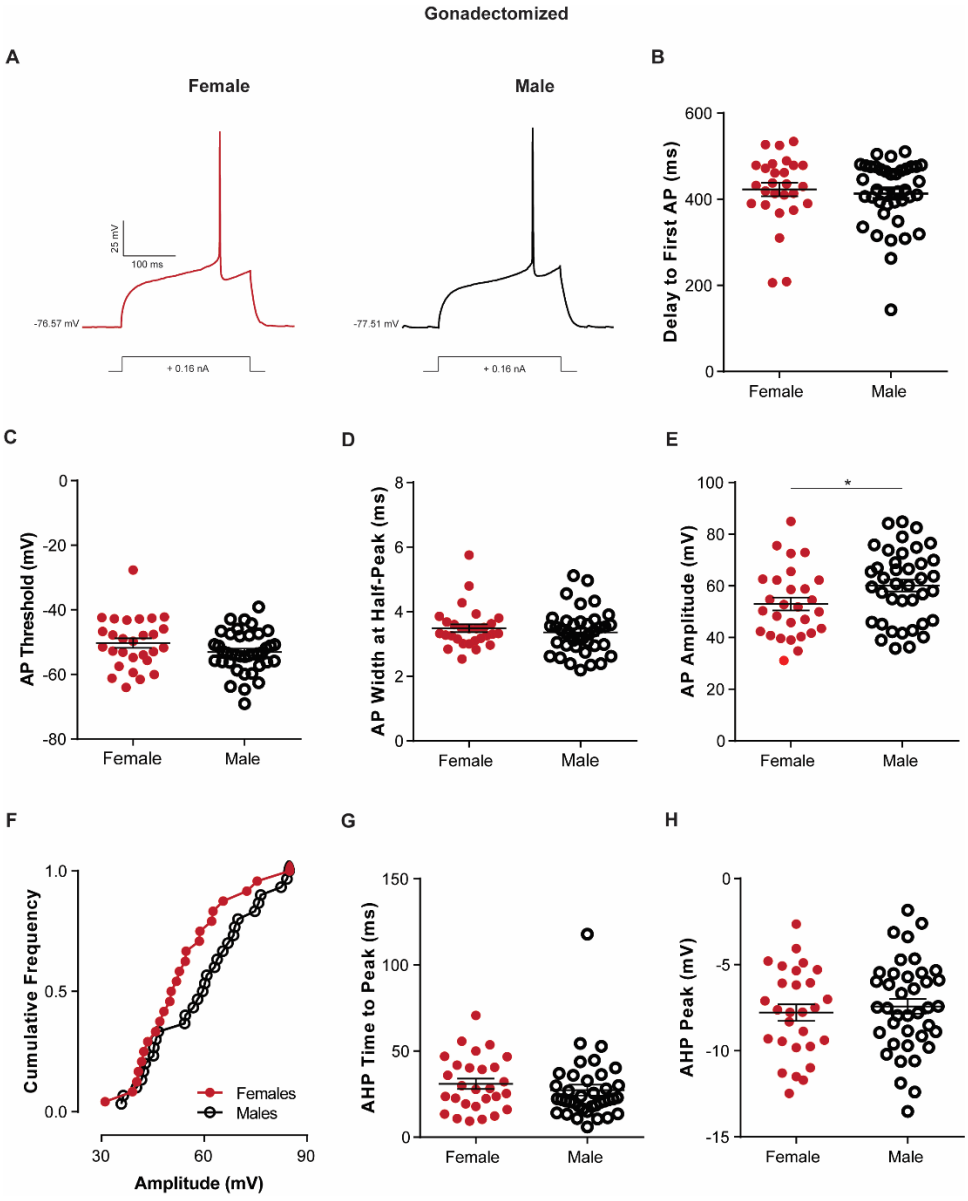

Figure 2.8. MSN individual action potential properties: gonadectomized females and males. A) Voltage responses of gonadectomized female and male MSNs to a single depolarizing current injection. The following action potential properties did not vary between gonadectomized females and males: B) delay to first action potential, C) action potential threshold, D) action potential width measured at half peak amplitude. The following action potential property appeared to vary between gonadectomized females and males: E) action potential amplitude. F) However, a cumulative frequency plot of action potential amplitude data distributions failed to reach significance, indicating that action potential amplitude is not a robust sex difference. The following action potential properties did not vary between gonadectomized females and males: G) action potential afterhyperpolarization time to peak amplitude, H) action potential afterhyperpolarization peak amplitude. The horizontal line superimposed upon scatter plots in Figures B through E, and G through H indicates the mean. The lines situated above scatter plots indicate statistical significance. Complete statistical information is in Table 2.5. Acronyms: AP, action potential; AHP, afterhyperpolarization.

The estrous cycle is necessary for sex differences in rheobase, the amount of current required for action potential initiation

In gonad-intact animals, MSN action potential initiation and generation varied across the estrous cycle, with MSNs recorded from diestrus phase females exhibiting decreased rheobase compared to MSNs recorded from proestrus females and males. To test whether this difference persisted in gonadectomized animals, we injected a series of positive current injections into MSNs and analyzed the resulting number of evoked action potentials (Figure 2.9A). We found that MSN action potential initiation and generation in the AcbC did not differ by sex in gonadectomized animals (Figure 2.9B). Consistent with this conclusion, no sex differences were detected in rheobase (Figure 2.9C) the slope of the linear range of the evoked firing rate per positive current injection curve (FI Slope) (Figure 2.9D), or minimum firing rate (Figure 2.9E). No sex difference was detected in resting membrane potential (Table 2.5). These findings do not exhibit a sex difference in gonadectomized rats, indicating that MSN action potential ignition and generation are regulated by the estrous cycle.

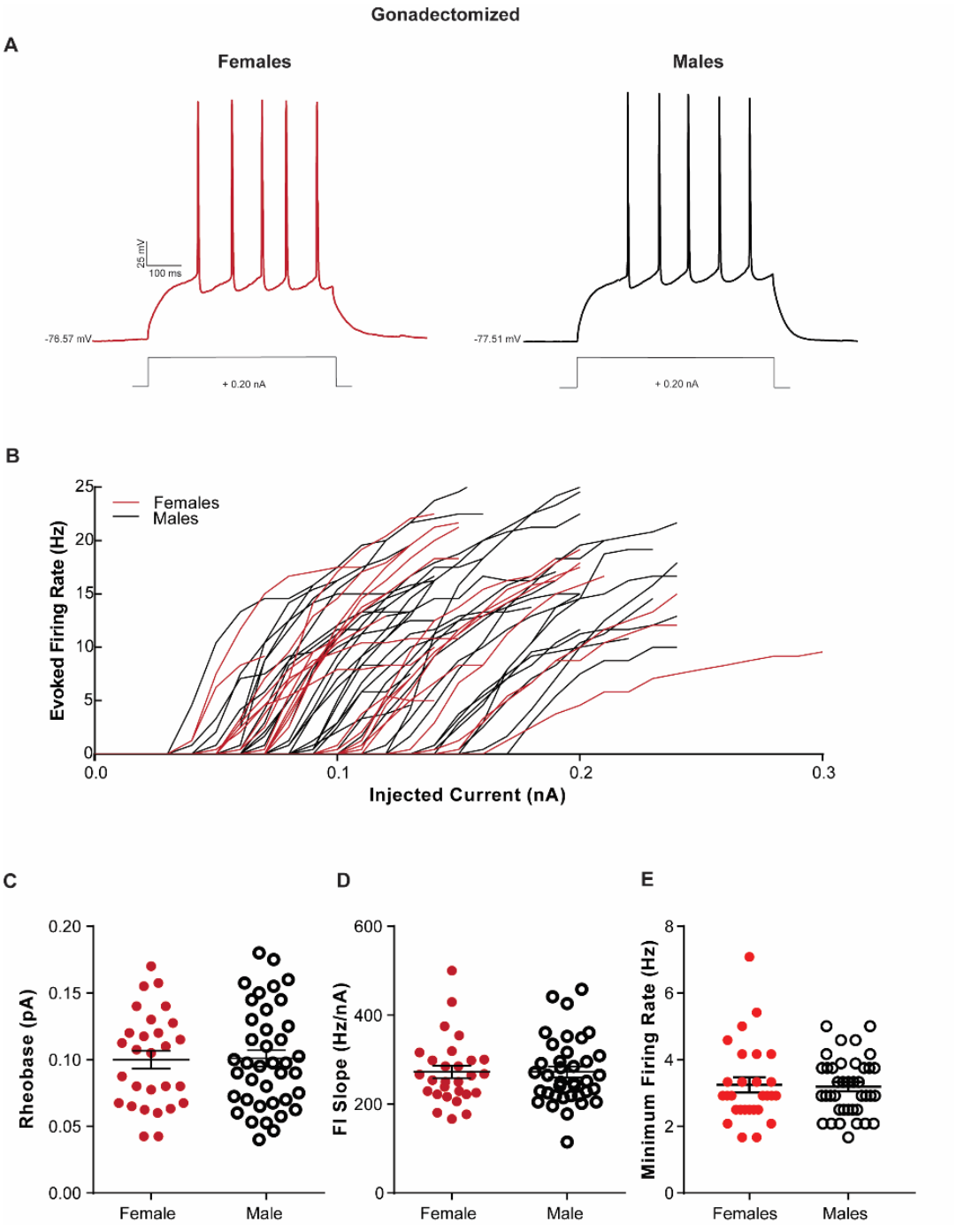

Figure 2.9. MSN action potential initiation and generation: gonadectomized females and males. A) Voltage responses of gonadectomized female and male MSNs to a series of depolarizing current injections. B) Action potential firing rates evoked by depolarizing current injections. The following properties did not vary between gonadectomized females and males: C) rheobase, D) the slopes of the evoked firing rate to positive current curve (FI Slope) in individual MSNs, E) minimum firing rate. The horizontal line superimposed upon scatter plots in Figures C through E indicates the mean. Complete statistical information is in Table 2.5. Acronyms: FI Slope, slope of the evoked firing rate to positive current curve.

Input resistance does not differ between gonadectomized females compared to males

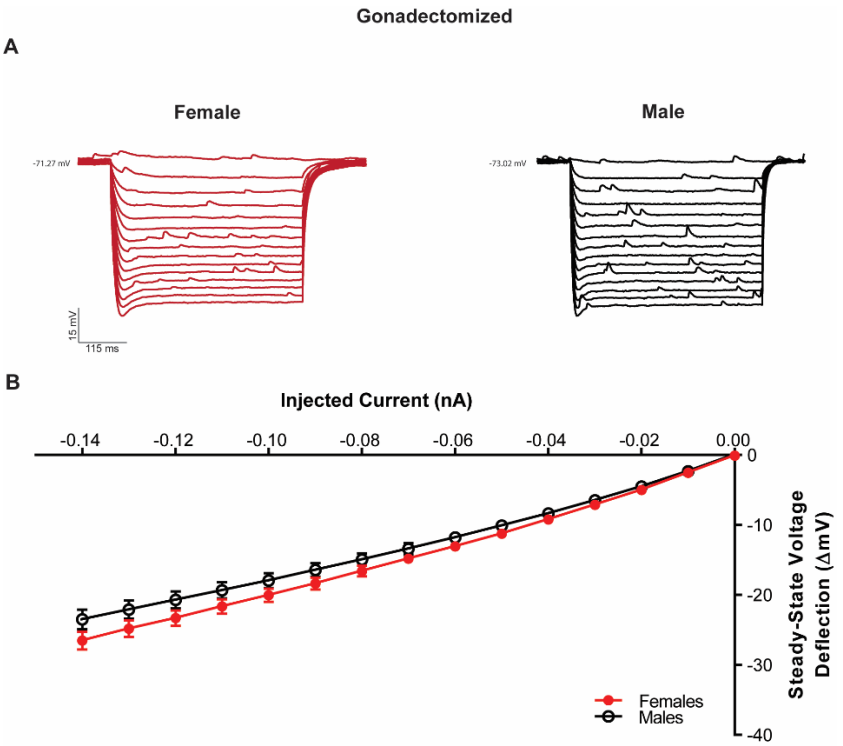

Figure 2.10. MSN passive electrophysiological properties: gonadectomized females and males. A) Voltage response of gonadectomized female and male MSNs to a series of hyperpolarizing current injections. B) The injected current to steady stage voltage deflection curve (IV curve) did not vary between gonadectomized females and to males.

Input resistance in diestrus phase females was increased compared to proestrus females and males (Figure 2.5A). To assess whether this difference is associated with the estrous cycle, we injected a series of negative current injections into gonadectomized female and male MSNs (Figure 2.10A). When the steady-state voltage deflection evoked by injected current curve (IV curve) was plotted, female MSNs showed no robust differences compared to male MSNs (Figure 2.10B). Consistent with this, input resistance in the linear range (Figure 2.11A) or rectified range (Figure 2.11B) did not differ by sex. Other passive membrane properties also did not differ by sex, including the time constant of the membrane (Figure 2.11C). In total, this collection of evidence indicates that input resistance does not differ between gonadectomized females and males, congruent with the hypothesis that input resistance is modulated by the estrous cycle.

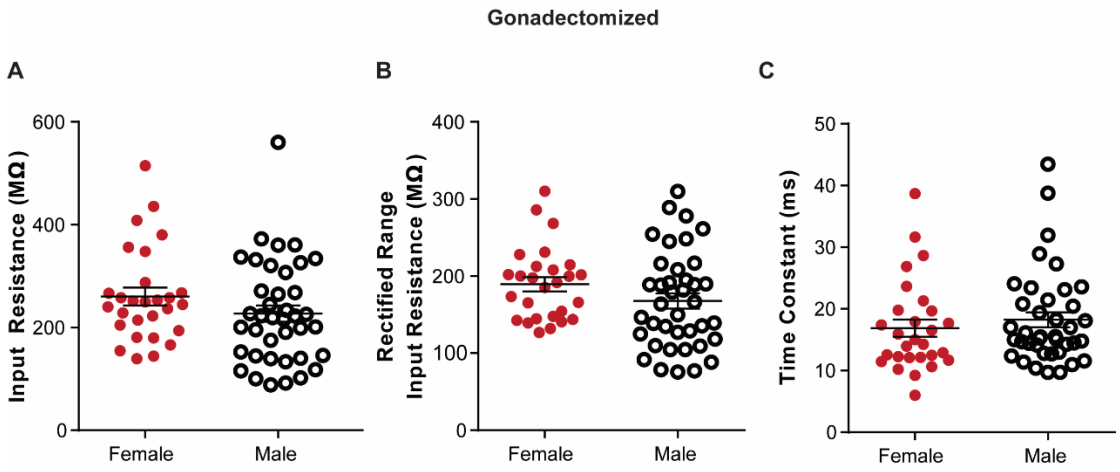

Figure 2.11. MSN input resistance and time constant of the membrane properties: gonadectomized females and males. The following properties did not vary between gonadectomized females and males: A) input resistance in the linear range, B) input resistance in the rectified range, C) time constant of the membrane. The horizontal line superimposed upon scatter plots in Figures A through C indicates the mean. Complete statistical information is in Table 2.5.

Table 2.5. Electrophysiological properties of MSNs in adult gonadectomized rat AcbC.

Property	Female	Male	Statistics (*t*/U, P)
Resting membrane potential (mV)	-86.32±0.60 (28)	-87.00±0.63 (39)	0.76, 0.45
Delay to first AP (ms)	422.90±15.63 (27)	413.20±12.26 (39)	475, 0.51*
Rheobase (nA)	0.10±0.01 (28)	0.10±0.01 (39)	0.09, 0.92
AP threshold (mV)	-50.30±1.49 (28)	-53.04±1.02 (39)	1.58, 0.12
AP width at half peak amplitude (ms)	3.49±0.12 (28)	3.36±0.11 (39)	495, 0.52*
AP amplitude (mV)	53.02±2.51 (28)[a]	60.11±2.24 (39)[b]	2.09, 0.04
AHP peak amplitude (mV)	-7.78±0.48 (28)	-7.42±0.43 (39)	0.56, 0.58
AHP time to peak (ms)	31.01±2.99 (28)	27.38±3.08 (38)	432, 0.20*
FI Slope (Hz/nA)	272.50±14.09 (28)	272.5±12.27 (37)	509, 0.91*
Time constant of the membrane (ms)	16.88±1.40 (28)	18.23±1.22 (39)	469, 0.33*
Linear range input resistance (MΩ)	260.20±17.2 (28)	227.2±15.94 (39)	420, 0.11*
Rectified range input resistance (MΩ)	189.40±9.11 (28)	167.80±10.03 (39)	1.51, 0.14
Percent inward rectification (%)	78.85±2.73 (28)	76.28±1.67 (39)	0.85, 0.40
Inward rectification (MΩ)	59.35±10.61 (28)	59.32±7.58 (39)	511, 0.66*
Sag Index (unitless)	0.008±0.003 (28)	0.006±0.001 (39)	485, 0.44*
Minimum Firing Rate (Hz)	3.24±0.23 (28)	3.19±0.14 (39)	0.13, 0.95

Notes: Values are mean ± SEM. Numbers in parentheses indicate number of neurons recorded. Shaded values indicate statistical significance. Acronyms: Action potential, AP; Afterhyperpolarization, AHP; Frequency of evoked action potentials to injected depolarizing current, FI. Superscript letters indicate statistically significant differences across groups. * denotes absence of normality.

Discussion

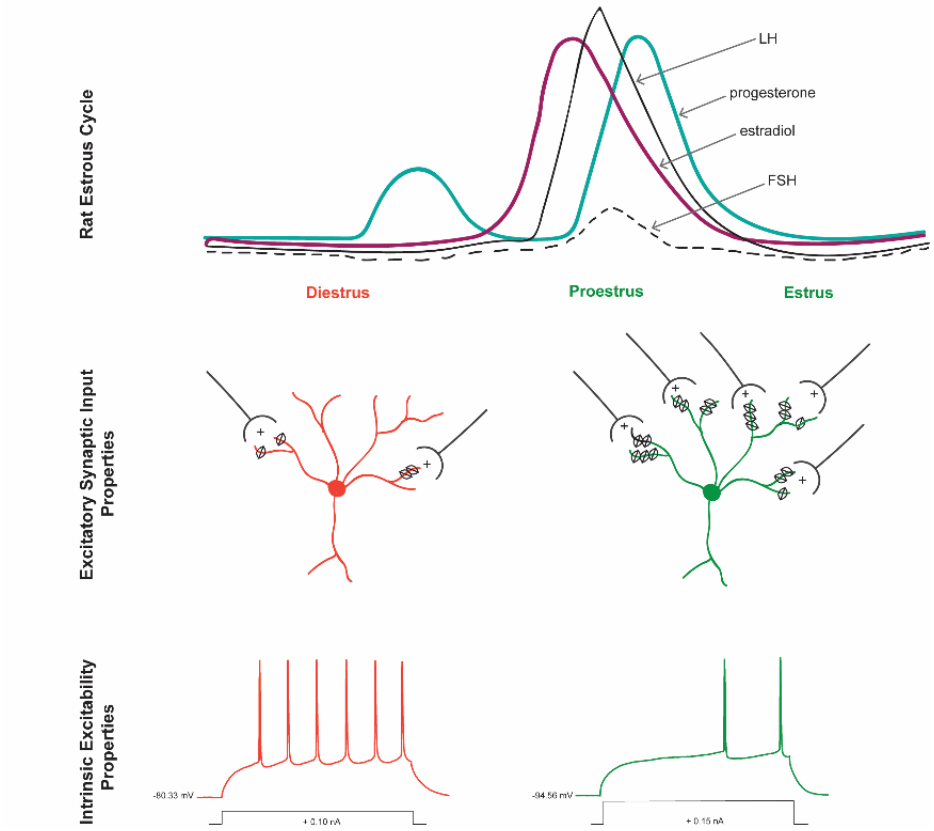

Figure 2.12. Schematic of AcbC MSN excitatory synaptic input and intrinsic excitability changes between estrous cycle phases in the adult female rat. Excitatory synaptic input properties are decreased in the diestrus phase of the cycle while intrinsic excitability is increased, as measured by changes in rheobase, resting membrane potential and input resistance. In the proestrus and estrus phases of the cycle, excitatory synaptic input is augmented, potentially through both an increase in excitatory synapse number and other presynaptic modifications, and postsynaptic AMPA receptor number and/or other properties. In contrast, intrinsic excitability is decreased.

This study demonstrates that MSN electrophysiological properties robustly change across the adult female estrous cycle, and, depending on the cycle phase, significantly differ from males as well (Figure 2.12). Specific discoveries include: 1) MSN excitatory synaptic input as assessed via mEPSC properties was augmented in females during the proestrus and estrus phases compared to females in diestrus phase and males; 2) Intrinsic neuronal excitability was decreased in females during proestrus and estrus compared to females in diestrus phase and males; 3) Removal of the estrous cycle via gonadectomy eliminated sex differences in MSN

electrophysiological characteristics. Collectively, these findings demonstrate that MSNs are highly sensitive to the estrous cycle, a natural, endogenous hormone cycle that ensures successful reproduction. These data provide a new framework for understanding how changes at the level of MSN electrophysiology potentially mediate hormone cycle influences on AcbC functions.

Interestingly, female MSNs in the proestrus and estrus phases show contrasting changes in excitatory synaptic input and intrinsic excitability. During proestrus and estrus, mEPSC frequency increases, as does mEPSC amplitude and decay, broadly indicating an augmentation of excitatory synaptic function. In contrast, during proestrus and estrus, resting membrane potential hyperpolarizes, action potential rheobase increases, and input resistance decreases. These attributes and others (Figure 2.12), indicate a decrease in intrinsic neuronal excitability, suggesting that large-scale changes in potassium or sodium ion channel distribution or function may be occurring in MSNs between estrous cycle phases. These findings are reminiscent of homeostatic plasticity, which encompasses electrophysiological phenomena, such as synaptic scaling, that stabilize individual neuron and neural circuit activity (Turrigiano 2012), that typically occur over long time periods such as days. Homeostatic plasticity has been demonstrated in MSNs in the nucleus accumbens (Ishikawa et al 2009). However, we hesitate to label the estrous-cycle induced changes in MSN function detected by this study as homeostatic plasticity, primarily because the precise hormone mechanism and temporal order of changes in MSN electrophysiological properties is unknown.

One mechanistic model that explains estrous cycle-induced changes in MSN electrophysiology is hormone- and activity-dependent. In this hormone- and activity-dependent model, during proestrus, sex steroid hormones act on excitatory synapses in the AcbC and

potentially its afferent brain regions to augment excitatory synaptic input onto MSNs. This augmentation of excitatory input includes, at a minimum, increase in excitatory synapse number, as elegant electron microscopy, immunocytochemistry, and electrophysiological studies have shown that excitatory synapse number increases in proestrus phase females compared to male rats (Forlano & Woolley 2010, Wissman et al 2012, Wissman et al 2011). Additionally, these glutamatergic synapses are sensitive to estradiol exposure in both adults and neonate rats as measured via mEPSC properties or dendritic spine density, a neuroanatomical correlate of excitatory synapse formation (Cao et al 2016, Martinez et al 2014, Peterson et al 2016, Peterson et al 2015, Staffend et al 2011). The experiments demonstrating estradiol-sensitivity in AcbC MSN dendritic spines detected that estradiol induced a decrease in dendritic spine density and that this effect was blocked by mGluR inhibitors and endocannabinoid pathways (Peterson et al 2016, Peterson et al 2015, Staffend et al 2011). At first glance, this appears contradictory to the current study's findings. However, in these studies, female rats were sacrificed 24 hours after exposure to estradiol, approximately placing these animals in a diestrus-like time period, long after estradiol levels have dropped and when mEPSC frequency is low. Thus, these results are consistent with the findings presented here.

The estradiol-sensitive changes observed in spine density reflect differences in excitatory synaptic transmission. Thus, increases in spine density could, in turn, induce differences in behavioral output such as locomotor activity and other behaviors influenced by the AcbC observed in proestrus phase female rats and other animal species, including humans during the follicular phase of the menstrual cycle (Jackson et al 2006). Over a period of hours, this increase in excitation, in turn, could then induce compensatory changes in MSN intrinsic excitability which ultimately reduces Acb-related locomotor behavior. This activity- and hormone-dependent

model is reminiscent of hormone-induced seasonal changes in songbird song control neuron electrophysiological and cellular anatomical properties in the robust nucleus of the arcopallium and the basal ganglia region Area X (Brenowitz & Remage-Healey 2016, Cohen et al 2016).

Alternatively, different hormones such as estradiol and progesterone could be synergistically acting to modulate specific aspects of MSN electrophysiology independent of hormone activity. For example, since dopaminergic terminals in nucleus accumbens MSNs express estrogen receptors such as GPER-1, membrane-associated estrogen receptor α, and membrane-associated estrogen receptor β (Almey et al 2015), estradiol alone may directly regulate both excitatory synapse properties and intrinsic properties. It is also possible that progesterone may also regulate changes in MSN electrophysiology as circulating plasma levels of progesterone also change with the estrous cycle. Consistent with this, progesterone has been demonstrated to modulate AcbC-mediated behaviors related to drugs of abuse (Becker 1999), and a membrane progesterone receptor has been identified in rat striatal tissue (Ke & Ramirez 1990, Ramirez et al 1996). Although nuclear progesterone and estrogen receptors are not detected in abundance in the AcbC, many brain regions which project to the AcbC express nuclear estrogen and progesterone receptors and could play an instrumental role in modulating MSN electrophysiology. We also note that testosterone may be acting in males to modulate AcbC properties since it has been documented that long-term testosterone exposure in males also modulates nucleus accumbens dendritic spine density (Wallin-Miller et al 2016).

The current experiments generate but cannot differentiate between these two, not necessarily mutually exclusive, models. It will be critical for future experiments to differentiate between early proestrus and late proestrus. More specifically, a future experiment will need to test the hypothesis as to whether changes in excitatory synapse properties occur during late

diestrus/early proestrus, when estradiol levels begin to rise, are unaccompanied by changes in intrinsic excitability. Furthermore, another critical next experiment will be to employ exogenous systemic exposures to estradiol, progesterone, and a combination of estradiol and progesterone in ovariectomized females to determine the relative contribution of each hormone towards inducing specific changes in MSN electrophysiological properties. Other future directions include establishing the MSN subtypes and AcbC interneurons which are sensitive to the estrous cycle, and testing whether estrous-cycle sensitivity is present in striatal regions beyond the AcbC. We suspect that this is the case. In adult caudate-putamen, for example, classic experiments published in the 1980s demonstrated that systemic estradiol exposure in ovariectomized adult female rats elevated *in vivo* spontaneous action potential generation and dopamine sensitivity (Arnauld et al 1981). Later experiments elucidated that nigrostriatal MSNs increased *in vivo* spontaneous action potential generation during proestrus and estrus phases in gonad-intact adult female rats (Tansey et al 1983), and in response to exogenous estradiol exposure in ovariectomized adult female rats. However, other MSN subtypes and striatal interneurons were not examined.

One difference between this study and two previous studies of sex differences in AcbC MSN regards mEPSC properties. Previous studies of adult MSNs in females of unreported estrous cycle phase and of pre-pubertal MSNs detected increased mEPSC frequency in females compared to males (Cao et al 2016, Wissman et al 2011), similar to the current study. However, these previous studies did not detect sex differences in mEPSC amplitude or decay, unlike the present study. Several possibilities exist that could explain these differences. First, previous experiments did not separate female animal data by estrous cycle and also, by necessity, employed animals exposed to either cocaine or vehicle injections prior to analysis, unlike the

present work (Wissman et al 2011). Second, the other relevant experiment employed pre-pubertal animals (Cao et al 2016). It is possible that activational hormone effects during puberty modulate the excitatory synaptic inputs onto the AcbC, reminiscent of changes in dopamine receptor complement in the AcbC during puberty (Andersen et al 2002, Brenhouse et al 2015, Teicher et al 1995). Further, the properties of excitatory synapse in the nucleus accumbens shell have been shown to be sensitive to the effects of environmental stimuli such as drugs of abuse and stress (Brancato et al 2017, Hodes et al 2015), reminiscent of classical research in the hippocampus (Shors et al 2001). While it remains formally possible that the lack of sex differences in the gonadectomized animals is because of surgical stress instead of hormone cycle removal, this explanation is unlikely because stress generally augments sex differences in excitatory synapse genetic markers (Brancato et al 2017), a finding consistent with the sex differences in incidence and phenotype of stress-linked disorders such as depression and anxiety (Altemus et al 2014).

We also acknowledge that the temperature of mEPSC recordings differed between the current study and previous studies. This study recorded mEPSC properties at an average of ~22°C while Wissman and colleagues recorded at ~34°C and Cao and colleagues recorded at ~28°C. Thus, it is possible that a higher recording temperature would have eliminated differences in mEPSC amplitude and decay. Housing conditions also differed between previous studies. Cao and colleagues and the current study both employed glass water bottles, BPA-free caging, soy-free animal diets, and corncob-free bedding to help mitigate the potential confounding influences of endocrine disruptors. Wissman and colleagues, on the other hand, do not report employing any of these specialized housing conditions. Thus, it is possible that uncontrolled exposure to endocrine disruptors may decrease or otherwise alter the magnitude of

naturally-occurring sex differences (Patisaul 2017). However, mitigating this speculation are the sex differences detected in mEPSC frequency, which are similar in magnitude across studies. In addition, the conditions under which mEPSCs were recorded (-70 mV, in the presence of PTX and TTX), ensure that AMPA-mediated activity encompasses the majority if not all mEPSC activity. This suggests that estrous-cycle induced changes in mEPSC properties are generated by a combination of underlying factors, including increases in excitatory synapse number (Cao et al 2016, Wissman et al 2011), along with potential changes in AMPA receptor number, composition, or phosphorylation, on the post-synaptic side of the synapse. Given that medium spiny neurons in the AcbC and other striatal regions express membrane-associated estrogen receptors, it is possible that hormones are acting in the AcbC to directly manipulate excitatory synapse function, as in other brain regions such as the hippocampus (Oberlander & Woolley 2016).

The present study focuses on the changes in circulating plasma sex steroid hormone levels across the estrous cycle. Sex steroid hormones can also be manufactured in the brain independent of gonad activity. While there are strong species differences in estradiol levels in the telencephalon, rat AcbC contains aromatase, and estradiol has been measured in the nucleus accumbens of ovariectomized rats (Morissette et al 1992). Furthermore, in the caudate-putamen, it has been reported that at least one form of LTP associated with excitatory synapse onto male MSNs is dependent on aromatase activity (Tozzi et al 2015). Thus, while gonadectomy and the consequent elimination of changes in circulating sex steroid hormones is sufficient for eliminating estrous-cycle induced sex differences in MSN electrophysiology, it remains possible that local hormone synthesis could be playing a role in other aspects of AcbC function. More broadly, given that environmental stimuli such as exposure to drugs of abuse can manipulate

other excitatory synapse properties such as NMDA/AMPA ratio (Andersen et al 2002, Brenhouse et al 2015, Jedynak et al 2016, Kourrich et al 2007, Rothwell et al 2011, Teicher et al 1995), perhaps many sex differences in AcbC synaptic physiology remain undiscovered. Collectively, these sex differences and hormone sensitivity may set the state for not only sex differences in AcbC-influenced behaviors, but also prepare this brain region for the reward-related activities related to reproduction (Bradley et al 2005).

Beyond the AcbC, it is notable that relatively few brain regions, much less neuron types, have been tested for estrous-cycle differences in neuron function (Alreja 2013, Blume et al 2017, Terasawa & Timiras 1968, Wang et al 2016, Woolley et al 1990). We suspect that many other neuron types and perhaps even glia, given their sensitivity to estradiol, also exhibit differential properties across cycle phases in sexually mature females. This potential has profound implications for our understanding of the plasticity inherent in neuron physiology in both females and males.

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CHAPTER 3

Differential and synergistic roles of 17β-estradiol and progesterone in modulating adult female rat nucleus accumbens core medium spiny neuron electrophysiology

Research in this chapter is under review with the following journal:

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Abstract

Naturally occurring cyclical changes in sex steroid hormones such as 17β-estradiol and progesterone can modulate neuron function and behavior in female mammals such as rats, mice, and humans. One example is the estrous cycle in rats, which is composed of multiple phases. We previously reported evidence of differences between estrous cycle phases in excitatory synapse and intrinsic electrophysiological properties of rat nucleus accumbens core (AcbC) medium spiny neurons (MSNs). The AcbC is a nexus between the limbic and premotor systems and is integral for controlling motivated and reward-associated behaviors and disorders, which are sensitive to the estrous cycle and hormones. The present study expands our prior findings by testing whether circulating levels of estradiol and progesterone correlate with changes in MSN electrophysiology across estrous cycle phases. As part of this project, the excitatory synapse and intrinsic excitability properties of MSNs in proestrus PM of adult female rats were assessed. Circulating levels of estradiol correlate with resting membrane potential, the time constant of the membrane, and rheobase. Circulating levels of progesterone correlate with mEPSC frequency and amplitude. Circulating levels of estradiol and progesterone together correlate with mEPSC amplitude, resting membrane potential, and input resistance. The proestrus PM phase features a prominent and unique decrease in mEPSC frequency. These data indicate that circulating levels of estradiol and progesterone alone or in combination interact with specific MSN electrophysiological properties, indicating differential and synergistic roles of these hormones. Broadly, these findings illustrate the underlying endocrine mechanism of how the estrous cycle modulates MSN electrophysiology.

Introduction

The menstrual cycle in humans and its analogous cycle in rodents, the estrous cycle, influence neuron physiology across multiple brain regions (Adams et al 2018, Blume et al 2017, Olmos et al 1989, Proano et al 2018, Terasawa & Timiras 1968, Willett et al 2019, Woolley & McEwen 1993). Both cycles feature cyclical changes in the circulating levels of ovarian hormones such as 17β-estradiol (estradiol) and progesterone and are divided into distinct phases. These phases are characterized by differing concentrations of estradiol and progesterone as well as by accompanying changes in reproductive organ physiology and associated reproductive behaviors (Beach 1976, Blaustein 2008, Erskine 1989, Hubscher et al 2005, Kow & Pfaff 1973, Micevych et al 2017, Westwood 2008). The rat estrous cycle typically occurs over a 4-5-day time period and is divided into distinct phases, including diestrus, proestrus, and estrus. Diestrus features relatively low levels of estradiol and progesterone that then gradually begin to rise. Proestrus is when these hormones reach peak circulating levels in a temporally distinct fashion. The morning of proestrus features a surge in circulating levels of estradiol, followed in the afternoon by a surge in circulating levels of progesterone. This differential hormone action, coupled with other physiological processes, allows the proestrus phase to be further subdivided into proestrus AM and proestrus PM phases (Adams et al 2018). The third phase is estrus, when the effects of estradiol and progesterone linger, even though the circulating concentrations are low. Recently, we showed that the excitatory synaptic input and intrinsic excitability properties of nucleus accumbens core (AcbC) medium spiny neurons (MSNs) robustly change across the estrous cycle (diestrus, proestrus AM, and estrus) (Proano et al 2018). These findings provided the first line of evidence in support of the documented changes in AcbC-mediated behaviors that differentially manifest across the estrous cycle in rodents and the menstrual cycle in humans.

These behaviors include those related to motivation, reward and reinforcement, and pathologies such as anxiety, depression, and addiction, which are all regulated by the AcbC (Becker & Hu 2008, Becker et al 2001, Evans & Foltin 2006, Lebron-Milad & Milad 2012, Milad et al 2009).

The AcbC is a highly conserved brain region that serves as a nexus between the limbic and premotor systems that manages the cognitive processing of motivation, reward, and reinforcement (Floresco 2015, Francis & Lobo 2017). It receives glutamatergic inputs from prefrontal cortex, amygdala, and hippocampus and dopaminergic inputs from the ventral tegmental area (Deroche et al 2020, Salgado & Kaplitt 2015), among others. These inputs converge and are then integrated by GABAergic MSNs, the primary output neuron subtype in the AcbC, and, by extension, are part of the reward circuitry of the brain (Salgado & Kaplitt 2015). Recent findings have shown that AcbC MSNs in adulthood express membrane-associated estrogen receptors α, β, and GPER-1 (Almey et al 2012, Almey et al 2015, Almey et al 2016). AcbC dopaminergic and glutamatergic action, such as synapse number, receptor number, and neurotransmitter availability exhibit sensitivity to estradiol (Becker 1999, Becker et al 2012, Calipari et al 2017, Forlano & Woolley 2010, Meisel & Mullins 2006, Mermelstein et al 1996, Wissman et al 2012). Furthermore, the AcbC is sensitive to developmental and acute estradiol action (Cao et al 2016, Cao et al 2018, Krentzel et al 2019, Krentzel et al 2020, Perry et al 2013, Tonn Eisinger et al 2018). In addition to the effects of estradiol, progesterone and its derivatives have been documented to modulate $GABA_A$ receptors by acting as chloride channels, albeit not yet in the AcbC (Backstrom et al 2014, Backstrom et al 2011). There is select evidence for progesterone receptor expression in the AcbC (Dluzen & Ramirez 1989b, Ke & Ramirez 1990, Piechota et al 2017, Sterling et al 1987).

The individual contributions of circulating levels of estradiol and progesterone in the modulation of excitatory synaptic input and intrinsic excitability properties across the estrous cycle in the AcbC is unknown. This is a critical knowledge gap given that the estrous cycle is a natural variable that not only induces robust neural and behavioral changes but is an integral part of adult female physiology of reproductive age. Here we address this question by testing the hypothesis that circulating levels of 17-β estradiol and progesterone, alone or in combination, correlate with changes in MSN electrophysiological properties across the estrous cycle. To accomplish this, we first augmented our existing dataset of female rat MSNs in diestrus, proestrus AM and estrus (Proano et al 2018) by conducting additional whole-cell patch clamp recordings of AcbC MSNs from proestrus PM, which has never before been performed by any laboratory group. We then employed a hormone extraction technique to measure 17-β estradiol and progesterone levels from blood serum samples obtained at sacrifice from rats in all phases of the estrous cycle. Using this expanded dataset, we then tested whether circulating levels of 17-β estradiol and progesterone correlated with MSN electrophysiological properties.

Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committees at North Carolina State University. Postnatal day 60 female Sprague-Dawley CD IGS rats were purchased from Charles River Laboratories. Rats were housed in pairs until postnatal day 65. After postnatal day 65, animals were individually housed to facilitate assessment of proestrus PM (n=12). Animals in other phases were generated in a recent and directly preceding study that employed the same methods as employed in the current study (Proano et al., 2018). New to this study are animals in proestrus PM. For the purposes of this

study, proestrus PM is defined as the time near the end of the ~12 hour proestrus phase, when vaginal cytology exhibits predominantly round and nucleated cells, with initial but minimal appearance of clumped cornified epithelial cells that is characteristic of the estrus phase. For the purpose of this study, proestrus AM is defined as near the beginning of the ~12-hour proestrus phase, when vaginal cytology exhibits only round and nucleated epithelial cells. Age at recording ranged from P70 to P85. All animals were housed in a temperature- and light-controlled room (23 °C, 40% humidity, 12:12-h light-dark cycle) at the Biological Resource Facility of North Carolina State University. All cages were washed with polysulfone Bisphenol A (BPA) free and were filled with bedding manufactured from virgin hardwood chips (Beta Chip; NEPCO, Warrensburg, NY) to avoid the presence of endocrine disruptors in corncob bedding (Mani et al 2005, Markaverich et al 2002, Villalon Landeros et al 2012). Soy protein-free rodent chow (2020X; Teklad, Madison, WI) and glass bottle-provided water were available *ad libitum*.

Animals were divided into two groups to capture the proestrus PM and to control for a possible circadian cycle effect. One group of animals (n=6) was exposed to a reverse light cycle with lights on at 7PM and off at 7AM and the other group (n=6) was exposed to a regular light cycle with lights turning on and off at 7AM and 7PM, respectively, as in our previous studies on the effects of the estrous cycle on rat AcbC and caudate-putamen (Proano et al 2018, Willett et al 2019). Estrous cycle assessment was performed beginning on P65 with a wet mount preparation as previously described (Hubscher et al 2005, Proano et al 2018). Females were vaginally swabbed at ~6:30 AM or ~6:30 PM, respectively. Slides were visualized under a microscope to determine estrous cycle stage. No statistically significant differences were detected between animals on a regular light cycle and those on a reverse light cycle. Thus, all data from proestrus PM are grouped for the remainder of the manuscript. Electrophysiological data from animals in

proestrus AM was collected from animals experiencing a regular light cycle and was previously published as described above.

Hormone extractions and assays

At sacrifice trunk blood was collected and centrifuged (within 30 min) at 4000 rpm for 35 minutes to obtain serum from 33 animals (diestrus: n=10; proestrus AM: n=7; proestrus PM: n=12; estrus: n=4). Serum was not available from 5 animals (diestrus: n=1; proestrus AM: n=1; estrus: n=3). Thus, electrophysiology data obtained from these animals was excluded from correlations analysis. Serum was stored at -80°C until extraction. Hormone extraction and enzyme-linked immunosorbent assay protocols were modified from previously described protocols (Chao et al 2011, Hedges et al 2018, Krentzel et al 2020, Tuscher et al 2016). Briefly, 250 µL of serum were extracted twice with a 10:1 ratio of diethyl ether followed by snap freeze with liquid nitrogen. The ether-containing organic compounds were poured off into clean glass tubes and dried overnight. This process was conducted twice. Once dry, the samples were resuspended in 200 µL of buffer from the enzyme-linked immunosorbent assay kit (Calbiochem Estradiol and Cayman Progesterone ELISA kits). Samples were measured in duplicates including extraction efficiency controls. Estradiol and progesterone were measured from the same samples. A 1:100 dilution of the sample was used for the progesterone assay and the remaining volume of the sample was used for the estradiol assay. Dilution factors were selected based on a dilution curve conducted prior to the experimental assay. Extraction efficiency for estradiol was 125% with blank at (Mean ± SE=2.840±0.271 pg/mL) and intra-assay variability was 14.45%; progesterone measurements were from same extracted samples as estradiol therefore they share the same extraction efficiency. Progesterone blank was at (Mean ±SE= 85.365±28.568 pg/mL) and intra-assay variability was 3.3%. All samples were above detectability for the assay. We note

that although both employed ELISA kits are advertised by the manufacturer as being able to detect hormone levels in unextracted serum, our experience is that detection levels are substantially decreased when unextracted serum is used. Progesterone levels significantly differed between estrous cycle phases (Table 3.1). Estradiol levels were elevated in proestrus AM and PM as *a priori* expected, however statistical significance was not reached, likely because of low power and the expected variability between groups (Table 3.1).

Table 3.1. Circulating levels of estradiol and progesterone across the estrous cycle.

Hormone	Diestrus	Proestrus AM	Proestrus PM	Estrus	Statistics (*F/BF, P*)
17β-estradiol, pg/mL	8.2±1.3 (10)	11.4±1.7 (7)	11.7±1.6 (12)	8.2±2.3 (4)	1.409, 0.269
Progesterone, ng/mL	34.8±4.8 (10)[a]	28.6±7.3 (7)[a]	51.3±6.1 (12)[b]	43.4±6.0 (4)[a, b]	3.173, 0.043

Values are means ± SE for numbers of animals in parentheses. Colored cells indicate statistical significance. [a, b] Superscript letters indicate statistically significant differences across groups. BF, Brown-Forsyth.

Acute brain slice preparation

Brain slices containing the nucleus accumbens core were prepared as previously described (Proano et al 2018). Animals were deeply anesthetized with isoflurane gas before decapitation (~7:00 AM or ~7:00 PM, for the reverse and regular light cycles, respectively; for the proestrus PM phase that is new to this study). The brain was rapidly extracted into ice-cold oxygenated sucrose artificial cerebrospinal fluid containing (in mM) 75 sucrose, 1.25 NaH_2PO_4, 3 $MgCl_2$, 0.5 $CaCl_2$, 2.4 Na pyruvate, and 1.3 ascorbic acid from Sigma-Aldrich (St. Louis, MO) and 75 NaCl, 25 $NaHCO_3$, 15 dextrose, and 2 KCl from Fisher (Pittsburgh, PA). The osmolarity of the sucrose ACSF was 295–305 mosM, and the pH was between 7.2 and 7.4. Coronal brain slices (300 m) were prepared with a vibratome and then incubated in regular ACSF containing (in mM) 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, and 2 $CaCl_2$ (295–305 mosM, pH 7.2–7.4) for 30 min at 30–35°C and then for at least 30 min at room

temperature (22–23°C). Slices were stored submerged in room-temperature oxygenated ACSF for up to 6 h after sectioning in a large-volume bath holder.

Electrophysiological recordings

Slices rested for at least 1 h after sectioning. They were then placed in a Zeiss Axioscope equipped with IR-DIC optics, a Dage IR-1000 video camera, and 10x and 40x lenses with optical zoom and superfused with oxygenated ACSF heated to ~21.63°C. Whole cell patch-clamp recordings were used to record the electrical properties of MSNs in the nucleus accumbens core (Figure 3.1). Glass electrodes contained (in mM) 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 $MgCl_2$, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine from Sigma-Aldrich and 10 HEPES from Fisher (285 mosM, pH 7.2–7.4). Signals were amplified, filtered (2 kHz), and digitized (10 kHz) with a MultiClamp 700B amplifier attached to a Digidata 1550 system and a personal computer using pCLAMP 10.7 software. Membrane potentials were corrected for a calculated liquid junction potential of 13.5 mV. As previously described (Dorris et al 2015), recordings were first made in current clamp to assess neuronal electrophysiological properties. MSNs were identified by medium-sized somas, the presence of a slow-ramping subthreshold depolarization in response to low-magnitude positive current injections, a hyperpolarized resting potential more negative than -65 mV, inward rectification, and prominent spike afterhyperpolarization (Belleau & Warren 2000, O'Donnell & Grace 1993). In a subset of recordings, oxygenated ACSF containing both the $GABA_A$ receptor antagonist picrotoxin (PTX, 150 M; Fisher) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 M; Abcam Biochemicals) was applied to the bath to abolish inhibitory postsynaptic current events and action potentials, respectively. Once depolarizing current injection no longer generated an action potential, MSNs were voltage clamped at 70 mV and mEPSCs were recorded for at least 5 min. These settings enable

recordings from almost exclusively AMPA glutamate receptors (Nowak et al 1984) and was confirmed by our laboratory in a previous study (Proano et al 2018). In all experiments, input/series resistance was monitored for changes, and cells were excluded if resistance changed 25%.

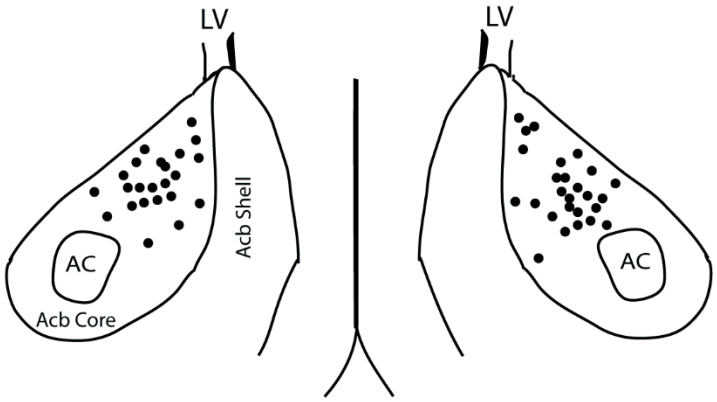

Figure 3.1. Location of proestrus PM whole cell patch-clamped MSNs in rat AcbC. Acb, nucleus accumbens; AC, anterior commissure; LV, lateral ventricle.

Data recording and analysis

Intrinsic electrophysiological properties and action potential characteristics were analyzed with pCLAMP 10.7. After break-in, the resting membrane potential was first allowed to stabilize ~1–2 min, as previously described (Mu et al 2010). After stabilization, resting membrane potential was assessed in the absence of injected current. At least three series of depolarizing and hyperpolarizing current injections were applied to elicit basic neurophysiological properties. Most properties measured followed definitions previously adopted by our laboratory (Cao et al 2016, Dorris et al 2015, Proano et al 2018, Willett et al 2019, Willett et al 2016) which were based on those of Perkel and colleagues (Farries et al 2005, Farries & Perkel 2000, Farries & Perkel 2002, Meitzen et al 2009). For each neuron, measurements were made of at least three action potentials generated from minimal current

injections. These measurements were then averaged to generate the reported action potential measurement for that neuron. For action potential measurements, only the first generated action potential was used unless more action potentials were required to meet the standard three action potentials per neuron. Action potential threshold was defined as the first point of sustained positive acceleration of voltage ($\delta^2V/\delta t^2$) that was also 3 times the SD of membrane noise before the detected threshold (Baufreton et al 2005). The delay to first action potential is the average time in milliseconds of the time from the initial deflection generated by the current step function to the action potential threshold of the first spike. Action potential width at half peak is the width of the action potential halfway between action potential peak and threshold in milliseconds. The action potential amplitude is the change in millivolts between action potential threshold and peak. Afterhyperpolarization peak amplitude is the difference in millivolts between action potential threshold and the most hyperpolarized voltage point after action potential peak. Afterhyperpolarization time to peak amplitude is the time measured in milliseconds between the action potential threshold voltage point on the descending phase of the action potential and the afterhyperpolarization peak amplitude. Rheobase, measured in nanoamps, is the lowest amplitude of injected positive current needed to produce an initial action potential. The slope of the linear range of the evoked action potential firing rate-to-positive injected current curve (FI slope) was calculated from the first current stimulus that evoked an action potential to the first current stimulus that generated an evoked firing rate that persisted for at least two consecutive current stimuli. Input resistance in the linear, non-rectified range was calculated from the steady-state membrane potential in response to 0.02-nA hyperpolarizing pulses. Rectified range input resistance, inward rectification, and percent inward rectification were calculated as described previously, with rectified range input resistance measured using the most hyperpolarizing current

injected into the MSN (Belleau & Warren 2000). Inward rectification is the input resistance of the 0.02-nA step minus the rectified range input resistance. Percent inward rectification is defined as rectified range input resistance/input resistance x 100. The time constant of the membrane was calculated by fitting a single exponential curve to the membrane potential change in response to 0.02-nA hyperpolarizing pulses. Possible differences in hyperpolarization induced "sag" were assessed with the "sag index" (Farries et al 2005). Briefly, the sag index is defined as the difference between the minimum voltage measured during the largest hyperpolarizing current pulse and the steady-state voltage deflection of that pulse, divided by the steady-state voltage deflection. A cell with no sag would exhibit a sag index of 0, whereas a cell whose maximum voltage deflection is twice that of the steady-state deflection would exhibit a sag index of 1. Cells with considerable sag typically have an index of 0.1. Frequency, amplitude, and decay of mEPSCs were analyzed off-line with Mini Analysis (Synaptosoft, http://www.synaptosoft.com/MiniAnalysis/). mEPSC threshold was set at a minimum value of 5 pA, and accurate event detection was validated by visual inspection. mEPSC frequency was defined as the number of detected mEPSC events per second (Hz). mEPSC amplitude was calculated as the difference between the averaged baseline 10 ms before initial mEPSC rise and peak mEPSC amplitude. mEPSC decay was calculated as the time required for peak mEPSC amplitude to return to baseline.

Statistics

Data were analyzed as appropriate with Pearson's linear regression, multiple linear regression, and a one-way ANOVA with Newman-Keuls and Brown-Forsyth post-hoc tests for parametric and nonparametric data sets, respectively (GraphPad Prism 8). P values < 0.05 were considered a priori as significant. Data are presented as means ± standard error.

Results

Here we test the central hypothesis that the electrophysiological properties of female rat AcbC MSNs correlate with circulating estradiol, progesterone, or both hormones. Our previously published study on estrous cycle effects on AcbC MSN electrophysiology was originally designed to solely capture proestrus AM (Proano et al 2018). However, to understand the individual contribution of estradiol and progesterone in modulating MSN electrophysiology, it was necessary to record and characterize electrophysiological properties of MSNs in proestrus PM as well. Thus, this results section is divided into two parts. Part I presents a novel characterization of proestrus PM AcbC MSN excitatory synapse and intrinsic excitability properties. These data are then combined with the previous dataset mentioned above to comprehensively analyze how MSN electrophysiological properties are modulated across the entire estrous cycle (Table 3.2). Using these data, part II then tests the hypotheses that circulating levels of estradiol, progesterone, and both estradiol and progesterone correlate with specific MSN electrophysiological properties.

Table 3.2. AcbC MSN electrophysiological properties.

Property	Diestrus	Proestrus AM	Proestrus PM	Estrus	Statistics (*F*/*NK*, *P*)
mEPSC frequency (Hz)	1.0±0.2 (19)[a]	2.1±0.4 (12)[b]	0.4±0.1 (25)[c]	2.3±0.4 (14)[b]	12.75, <0.0001
mEPSC amplitude (pA)	10.4±0.7 (19)[a]	11.40±0.9 (12)[a]	15.8±0.5 (25)[b]	9.1±0.3 (14)[a]	28.36, <0.0001
mEPSC decay (ms)	4.2±0.5 (19)[a]	4.8±0.5 (12)[a]	3.7±0.2 (25)[a]	2.7±0.3 (14)[b]	4.35, 0.0074
Resting membrane potential (mV)	-83.8±1.1 (26)[b]	-87.4±0.9 (20)[a]	-88.2±0.4 (49)[a]	-88.1±1.5 (16)[a]	6.40, 0.0005
Input resistance (MΩ)	299.1±18.7 (26)[a]	203.9±16.0 (20)[b, c]	209.4±8.4 (49)[b, c]	258.6±29.4 (16)[a, b]	8.53, <0.0001
Rectified range input resistance (MΩ)	217.5±12.7 (26)[a]	164.8±12.6 (20)[b, c]	169.5±6.4 (49)[b, c]	189.5±17.9 (16)[a, b]	4.95, 0.003
Inward rectification (MΩ)	81.7±14.1 (26)[a]	39.0±5.9 (20)[b, c]	39.9±3.8 (49)[b, d]	69.1±19.0 (16)[a, b]	5.13, 0.002
% inward rectification (%)	74.9±3.0 (26)	81.4±2.0 (20)	81.6±1.2 (49)	77.9±4.1 (16)	2.03, 0.115
Sag index (unitless)	0.004±0.004 (26)	0.003±0.001 (20)	0.010±0.002 (49)	0.005±0.004 (16)	1.51, 0.216
Time constant of the membrane (ms)	19.1±1.4 (26)[a]	14.8±1.2 (20)[a, b]	13.8±0.7 (49)[b, c]	16.4±2.4 (16)[a, b, c]	4.05, 0.009
Capacitance (pF)	68.1±6.1 (26)	78.1±7.4 (20)	68.8±3.4 (49)	65.4±6.4 (16)	0.78, 0.505
Rheobase (pA)	0.1±0.01 (26)[a]	0.1±0.01 (19)[b]	0.1±0.01 (49)[b]	0.1±0.01 (16)[b]	6.58, 0.0004
Delay to first AP (ms)	402.3±15.6 (18)[a]	438.9±13.2 (18)[a, c]	441.4±9.3 (48)[a, c]	465.9±14.6 (15)[b, c]	3.03, 0.033
AP threshold (mV)	-51.8±1.9 (26)	-47.4±2.0 (19)	-47.4±1.0 (49)	-50.5±2.0 (16)	2.06, 0.110
AP amplitude (mV)	54.8±3.0 (26)	54.6±3.5 (19)	48.3±1.6 (49)	55.0±3.5 (16)	2.18, 0.095
AP width at half-peak amplitude (ms)	3.8±0.2 (26)[a]	3.4±0.1 (17)[a]	4.0±0.1 (49)[b]	3.6±0.2 (16)[a, b]	3.80, 0.013
AHP peak amplitude (mV)	-7.2±0.5 (25)	-7.7±0.6 (19)	-9.0±0.4 (49)	-7.1±0.5 (16)	3.97, 0.010
AHP time to peak (ms)	25.8±3.1 (25)	23.2±2.1 (19)	25.7±1.5 (49)	26.3±3.3 (16)	0.26, 0.854
FI Slope (Hz/nA)	298.7±17.9 (26)[a]	241.3±14.8 (19)[b]	238.5±7.1 (49)[b]	297.1±21.2 (16)[a]	6.22, 0.001

Excitatory synaptic input and intrinsic excitability properties recorded from diestrus, proestrus AM, proestrus PM, and estrus medium spiny neurons in gonad-intact adult rat nucleus accumbens core. Values are means ± SE for numbers of animals in parentheses. Colored cells indicate statistical significance. [a, b, c, d] Superscript letters indicate statistically significant differences across groups. AP, action potential; AHP, afterhyperpolarization; FI, evoked firing rate-to-positive current curve. NK, Newman-Keuls.

Part I: proestrus PM

mEPSC frequency is drastically decreased in proestrus PM

To assess mEPSC properties we voltage clamped MSNs to -70 mV and applied 1 μM TTX and 150 μM PTX solution to block sodium-dependent action potentials and $GABA_A$ receptors, respectively (Figure 3.2A). We assessed mEPSC frequency, amplitude, and decay (Table 3.2). mEPSC frequency (Figure 3.2B) levels are remarkably low, ranging from about 0 to 1 Hz. mEPSC amplitude (Figure 3.2C) values range from around 10 to 22 pA while mEPSC decay (Figure 3.2D) levels average about 5 ms. When compared to previous description from other stages of the estrous cycle (Proano et al 2018), these findings demonstrate that excitatory synapse properties in proestrus PM MSNs show a drastic reduction in mEPSC frequency with a relatively robust increase in amplitude for the existing mEPSC events.

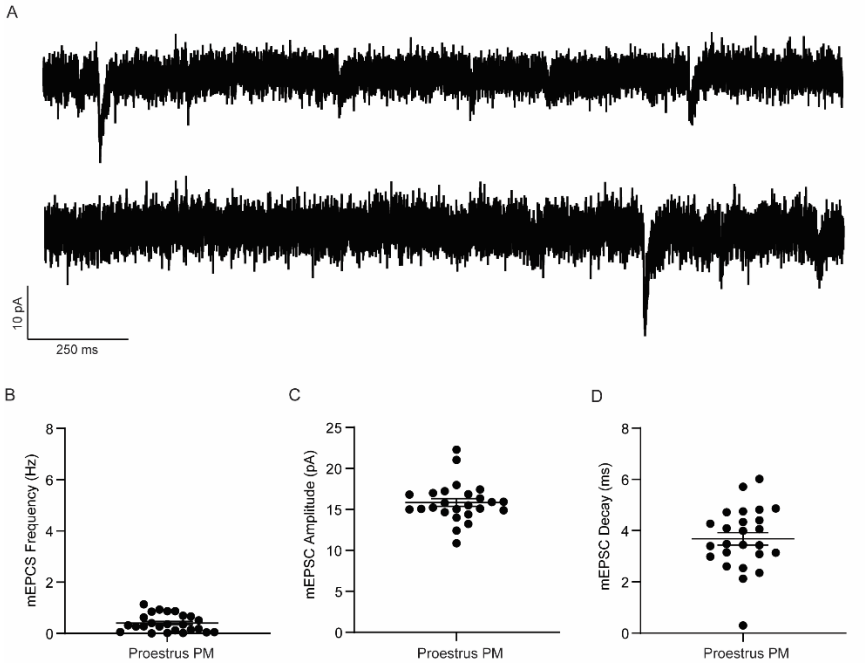

Figure 3.2. MSN miniature excitatory postsynaptic current (mEPSC) properties from proestrus PM females. *A*. Representative examples of mEPSCs recorded in proestrus PM. *B*. mEPSC frequency. *C*. mEPSC amplitude *D*. mEPSC decay. Horizontal line superimposed upon scatterplots in *B-D* indicates the mean.

Membrane excitability and passive membrane properties in proestrus PM

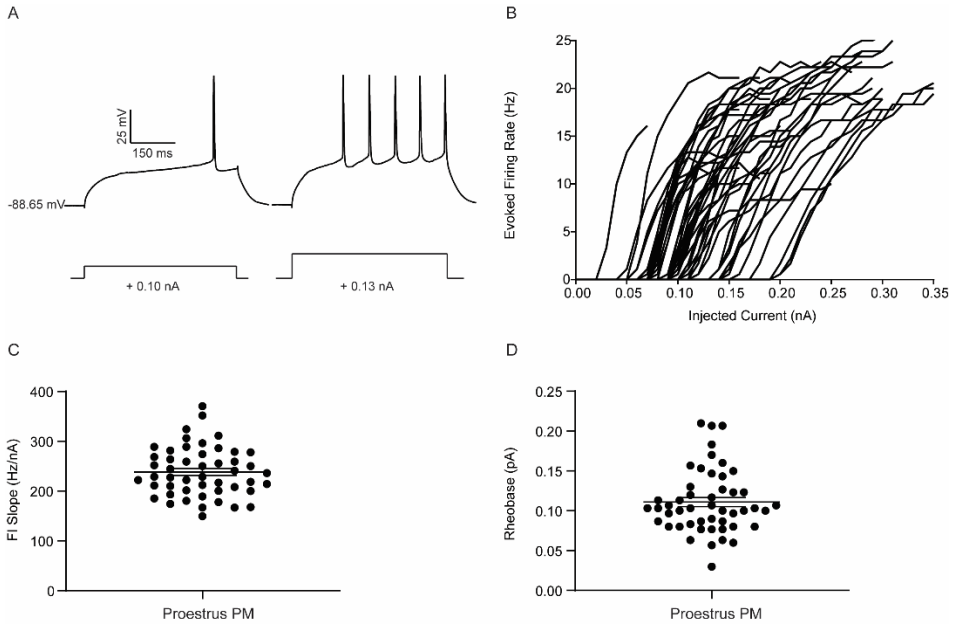

Figure 3.3. MSN action potential initiation and propagation properties in proestrus PM females. *A*. Voltage response of proestrus PM female MSNs to a series of depolarizing current injections. *B*. Action potential firing rates evoked by depolarizing current injections. *C*. Slopes of the evoked firing rate-to-positive current curve (FI slope) in individual MSNs. *D*. Rheobase. Horizontal line superimposed upon scatterplots in *C* and *D* indicates the mean.

We also assessed intrinsic electrophysiological properties in proestrus PM MSNs, including individual action potential properties, excitability, and passive membrane properties. To accomplish this, we injected MSNs with a series of depolarizing and hyperpolarizing current injections after which we analyzed an array of electrophysiological attributes. All electrophysiological attributes and related statistical information are provided in Table 3.2. Here we highlight excitability and passive membrane properties. Membrane excitability was assessed by injecting a series of depolarizing current injections into individual MSNs to quantify action potential initiation and propagation (Figure 3.3A). We first plotted the number of action potentials evoked by depolarizing current injection curve (FI curve) for individual MSNs (Figure 3.3B). Using these data, we quantified individual MSN excitability by calculating the slope of the evoked firing rate to positive current curve (FI Slope; Figure 3.3C). We also assessed

rheobase, which is the minimum amount of current required to initiate an action potential (Figure 3.3D). Regarding passive membrane properties, a series of hyperpolarizing current injections were administered to MSNSs (Figure 3.4A). A plot of the steady-state voltage deflection evoked by injected hyperpolarizing current curve (IV curve; Figure 3.4B) reveals inward rectification, as expected from previous studies of MSNs (Belleau & Warren 2000). We then quantified input resistance in the linear (Figure 3.4C) and rectified ranged (Figure 3.4D). These findings indicate that the excitability and passive membrane properties of proestrus PM MSNs fall within an expected range from previous studies except for mEPSC frequency. As a whole, these data enable a more complete analysis of AcbC MSN properties across the estrous cycle (Table 3.2).

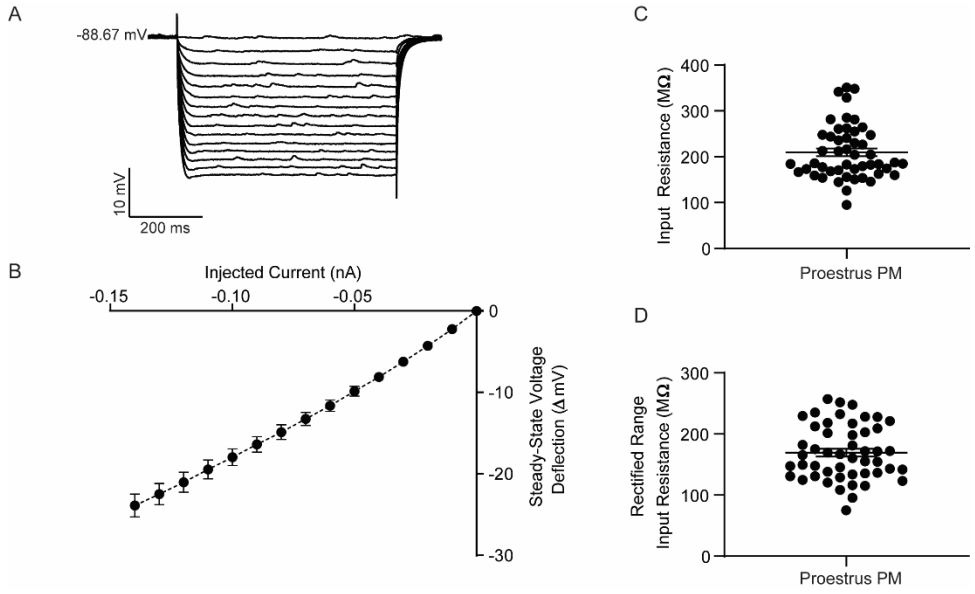

Figure 3.4. MSN passive electrophysiological properties in proestrus PM females. *A*. Voltage response of proestrus PM female MSNs to a series of hyperpolarizing current injections. *B*. The injected current-to-steady-stage voltage deflection curve (IV curve). *C*. Input resistance in the linear range. *D*. Input resistance in the rectified range. Horizontal line superimposed upon scatterplots in *C* and *D* indicates the mean.

Part II: circulating estradiol and progesterone levels, and MSN electrophysiology

The rat estrous cycle features cyclical fluctuations of estradiol and progesterone over a four- to five-day period. It can be divided into multiple phases, including the diestrus, proestrus AM, proestrus PM, and estrus phases. Our previous study demonstrated robust changes in adult

female AcbC MSN electrophysiological properties across the estrous cycle, which were abolished upon gonadectomy (Proano et al 2018). Our previous study did not analyze whether the electrophysiological properties of MSNs varied with circulating levels of estradiol or progesterone. This is notable gap in knowledge, as answering this question may provide clues towards the underlying endocrine mechanisms. Thus, here we test the hypothesis that circulating levels of estradiol, progesterone, and both estradiol and progesterone, correlate with specific MSN electrophysiological properties.

Table 3.3. Correlations between estradiol and progesterone with AcbC MSN electrophysiological properties.

	Correlations (r, p-value)		
Property	**Estradiol**	**Progesterone**	**Estradiol + Progesterone**
mEPSC frequency (Hz)	-0.01, 0.93	-0.30, 0.03	0.23, 0.10
mEPSC amplitude (pA)	0.06, 0.70	0.35, 0.01	0.28, 0.04
mEPSC decay (ms)	-0.001, 0.99	0.08, 0.57	0.07, 0.65
Resting membrane potential (mV)	-0.25, 0.02	-0.12, 0.28	0.24, 0.02
Input resistance (MΩ)	-0.20, 0.05	-0.19, 0.08	0.26, 0.01
Rectified range input resistance (MΩ)	-0.16, 0.12	-0.12, 0.24	0.22, 0.04
Inward rectification (MΩ)	-0.17, 0.12	-0.18, 0.09	0.20, 0.05
% inward rectification (%)	0.15, 0.16	0.11, 0.32	0.13, 0.22
Sag index (unitless)	0.03, 0.76	0.04, 0.68	0.05, 0.62
Time constant of the membrane (ms)	-0.21, 0.04	-0.12, 0.28	0.18, 0.09
Capacitance (pF)	0.03, 0.77	0.03, 0.78	0.08, 0.46
Rheobase (pA)	0.28, 0.01	-0.02, 0.85	0.18, 0.09
Delay to first AP (ms)	0.21, 0.06	0.06, 0.60	0.12, 0.27
AP threshold (mV)	0.14, 0.18	0.02, 0.84	0.09, 0.38
AP amplitude (mV)	0.04, 0.70	-0.06, 0.57	0.03, 0.77
AP width at half-peak amplitude (ms)	-0.01, 0.95	-0.01, 0.90	0.01, 0.93
AHP peak amplitude (mV)	0.05, 0.64	-0.04, 0.68	0.04, 0.74
AHP time to peak (ms)	-0.15, 0.15	-0.09, 0.39	0.16, 0.14
FI slope (Hz/nA)	-0.14, 0.20	0.03, 0.76	0.01, 0.35

Relationship between estradiol or progesterone and estradiol plus progesterone with excitatory synaptic input and intrinsic excitability properties of medium spiny neurons in gonad-intact adult rat nucleus accumbens core across the estrous cycle. Colored cells indicate statistical significance. AP, action potential; AHP, afterhyperpolarization; FI, evoked firing rate-to-positive current curve.

Circulating estradiol levels correlate with resting membrane potential, the time constant of the membrane, and rheobase

To test the hypothesis that circulating levels of estradiol correlate with specific MSN electrophysiological properties, we tested whether each animal's circulating estradiol levels correlated with all the MSN properties collected across all phases of the estrous cycle (Figures 3.5A and 3.5B). Three electrophysiological properties significantly correlate with circulating estradiol levels: resting membrane potential, the time constant of the membrane, and rheobase (Figure 3.5B). Complete statistical information is provided in Table 3.3. Resting membrane potential exhibits an inverse correlation with increasing circulating levels of estradiol across the estrous cycle (Figure 3.5C), suggesting that as estradiol levels increase, resting membrane potential decreases. If this relationship is robust, then the estrous cycle phases that feature higher circulating estradiol levels would exhibit hyperpolarized resting membrane potentials. Supporting this, resting membrane potential is significantly hyperpolarized in both proestrus AM and PM compared to the diestrus phase (Figure 3.5D). Interestingly, resting membrane potential remains hyperpolarized in the estrous phase compared to the diestrus phase, even though circulating levels of estradiol are decreasing in this phase. This may indicate that MSNs have yet to revert to a non-estradiol influenced state, as well as a potential role for a synergistic interaction between estradiol and progesterone, which is addressed below. The time constant of the membrane exhibits an inverse correlation with increasing levels of circulating estradiol (Figure 3.5E), suggesting that as estradiol levels increase, the time constant of the membrane decreases. To support this, the time constant of the membrane decreases during proestrus PM phase compared to the diestrus phase (Figure 3.5F). Rheobase features a positive correlation with increasing circulating estradiol levels (Figure 3.5G). This finding indicates that the minimum

amount of current required to initiate the first action potential increases as circulating levels of estradiol increase. If this correlation is robust, then we would expect that the estrous cycle phases that feature higher circulating estradiol levels would exhibit increased rheobase values. Consistent with this logic, rheobase is significantly elevated in proestrus AM and PM compared to the diestrus phase (Figure 3.5H). Rheobase is also significantly increased in the estrus phase compared to the diestrus phase, again suggesting a possible remaining impact of estradiol. Overall, these findings indicate that circulating levels of estradiol associate with resting membrane potential and rheobase. The association between estradiol, resting membrane potential, and rheobase is logical given that a more hyperpolarized resting membrane potential would require increasing rheobase values to drive the neuron towards threshold and fire an action potential. Thus, increasing circulating estradiol levels decrease the overall intrinsic excitability of AcbC MSNs.

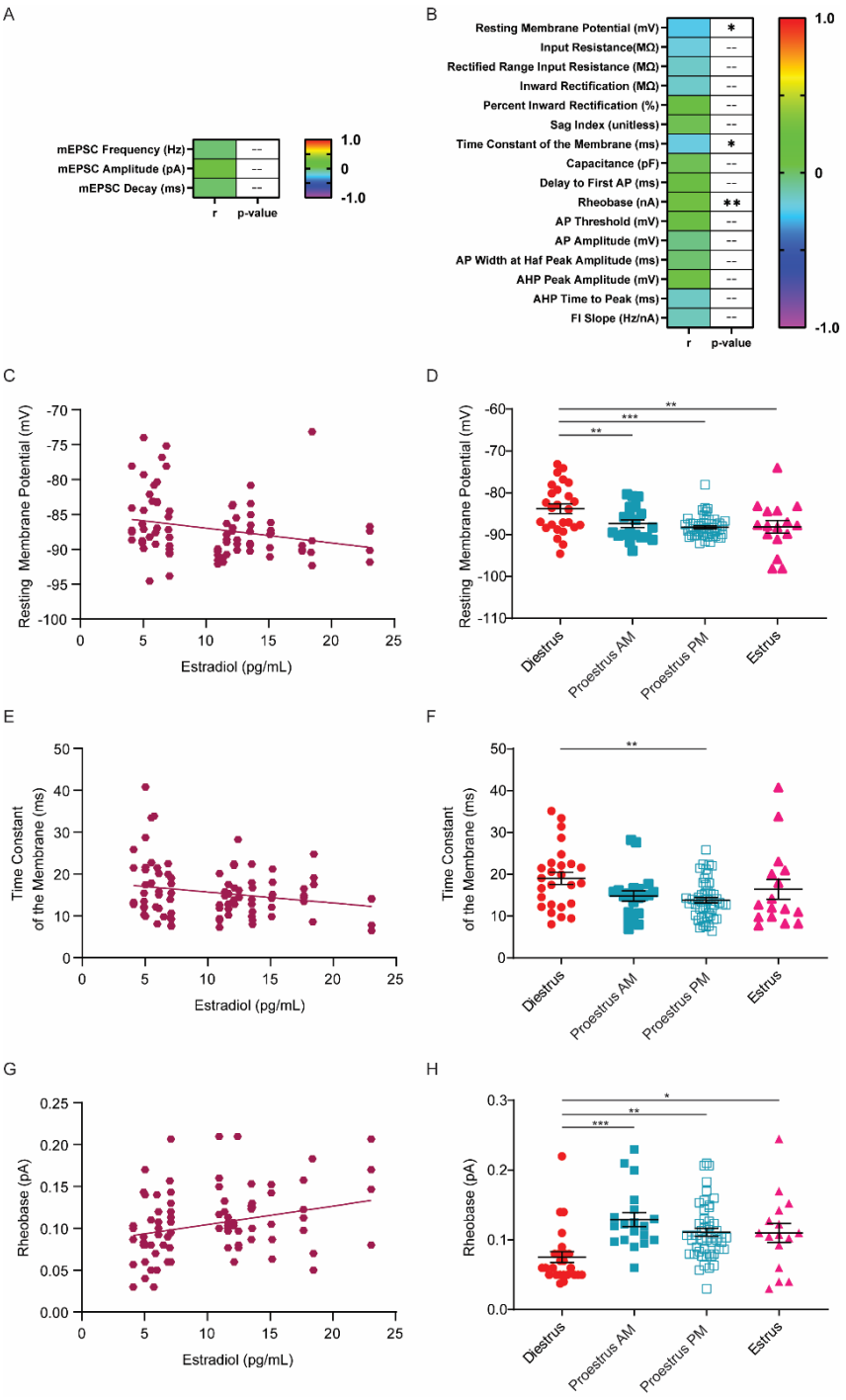

Figure 3.5. Correlations of electrophysiological properties across all phases of the estrous cycle with circulating levels of estradiol. *A*. Heat map of correlations of miniature excitatory post synaptic current (mEPSC) properties with circulating levels of estradiol. *B*. Heat Map of correlation of intrinsic excitability properties with circulating levels of estradiol. Statistically significant correlations in *A* and *B* are designated with *. *C* and *E*: resting membrane potential *(C)* and time constant of the membrane inversely correlate with circulating levels of estradiol. *G*. Rheobase displays a positive correlation with circulating levels of estradiol. *D*, *F*, and *H*: resting membrane potential *(D)*, time constant of the membrane *(F)*, and rheobase *(H)* vary across estrous cycle phase. Horizontal line superimposed upon scatterplots in *D*, *F*, and *H* indicates the mean. Lines above the scatterplots indicate statistical significance. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Circulating progesterone levels correlate with mEPSC frequency and mEPSC amplitude

To test the hypothesis that circulating levels of progesterone correlate with specific MSN electrophysiological properties, we tested whether each animal's circulating progesterone levels correlated with all the MSN properties collected across all phases of the estrous cycle (Figures 3.6A and 3.6B). Two electrophysiological properties significantly correlate with circulating progesterone levels: mEPSC frequency and mEPSC amplitude (Figure 3.6A). Complete statistical information is provided in Table 3.3. mEPSC frequency exhibits an inverse correlation with increasing circulating levels of progesterone (Figure 3.6C). This finding indicates that mEPSC frequency decreases as progesterone levels increase. If this correlation is robust, then we would expect that the estrous cycle phases that feature higher circulating levels of progesterone would exhibit a decrease in mEPSC frequency. Consistent with this rationale, mEPSC frequency is significantly decreased in proestrus PM compared to proestrus AM and estrus phases (Figure 3.6D). Interestingly, mEPSC frequency rebounds during the estrus phase, suggesting a possible acute effect of progesterone or estradiol. mEPSC amplitude features a positive correlation with increasing circulating levels of progesterone (Figure 3.6E), indicating that mEPSC amplitude increases as progesterone levels increase. If this is a robust correlation, then we would expect that estrous cycle phases that feature higher circulating progesterone levels would exhibit increased mEPSC amplitude. Consistent with this, mEPSC amplitude is significantly increased in proestrus PM compared to the diestrus, proestrus AM, and estrus phases of the cycle (Figure 3.6F). Interestingly, the variance of mEPSC amplitude values decreases radically during the estrus phase, suggesting a potential synergistic action between estradiol and progesterone. Overall, this analysis indicates that increasing circulating levels of progesterone alter MSN excitatory synapse properties.

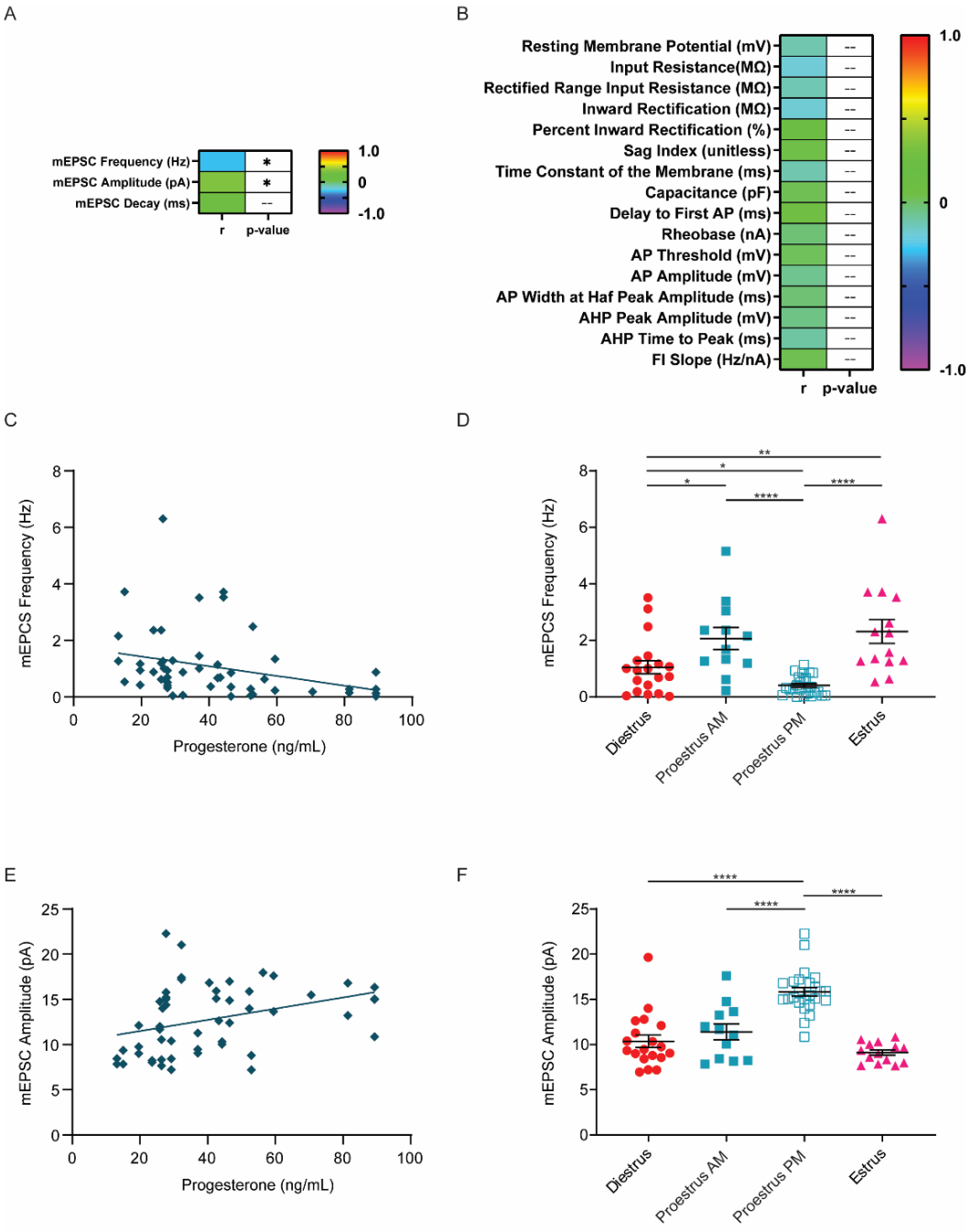

Figure 3.6. Correlation of electrophysiological properties across all phases of the estrous cycle with circulating levels of progesterone. *A*. Heat map of correlation of miniature excitatory post synaptic current (mEPSC) properties with circulating levels of progesterone. *B*. Heat map correlation of intrinsic excitability properties with circulating levels of progesterone. Statistically significant correlations in *A* and *B* are designated with *. *C*. mEPSC frequency inversely correlates with circulating levels of progesterone. *E*. mEPSC amplitude shows a positive correlation with circulating levels of progesterone. *D* and *F*: mEPSC frequency *(D)* and amplitude *(F)* vary across estrous cycle phase. Horizontal line superimposed upon scatterplots in *D* and *F* indicates the mean. Lines above the scatterplots indicate statistical significance. **P<0.05, **P<0.01, ***P< 0.001, ****P<0.0001*.

Circulating estradiol and progesterone levels together correlate with mEPSC amplitude, resting membrane potential, input resistance, and rectified range input resistance

To test the hypothesis that circulating levels of estradiol and progesterone together correlate with specific MSN electrophysiological properties, we tested whether each animal's circulating estradiol and progesterone levels correlate with all the MSN properties collected across all phases of the estrous cycle using a multiple linear regression with estradiol and progesterone as two independent variables. Four electrophysiological properties significantly correlate with circulating estradiol and progesterone levels: resting membrane potential, input resistance, rectified range input resistance, and mEPSC amplitude (Figures 3.7A and 3.7B). Complete statistical information is provided in Table 3.3. Resting membrane potential correlates with the interaction between circulating levels of estradiol and progesterone. Consistent with this finding, resting membrane potential remains hyperpolarized in proestrus PM and estrus phases compared to the diestrus phase, when the effects of both estradiol and progesterone would potentially manifest (Figure 3.5D). Input resistance in the linear range correlate with the interaction between circulating levels of estradiol and progesterone. Supporting this finding, input resistance is significantly lower in proestrus AM and PM compared to the diestrus phase, with intermediate values in the estrus phase (Figure 3.7C). Input resistance in the rectified range also correlates with the interaction between circulating levels of estradiol and progesterone. Accordingly, rectified range input resistance is significantly decreased in proestrus AM and PM compared to the diestrus phase, and again exhibits intermediate values in the estrus phase (Figure 3.7D). mEPSC amplitude also correlates with the interaction between circulating levels of estradiol and progesterone (Figure 3.7E). As expected, mEPSC amplitude begins to increase in

proestrus AM when circulating levels of estradiol increase and is significantly increased in proestrus PM when circulating levels of progesterone increase (Figure 3.6F).

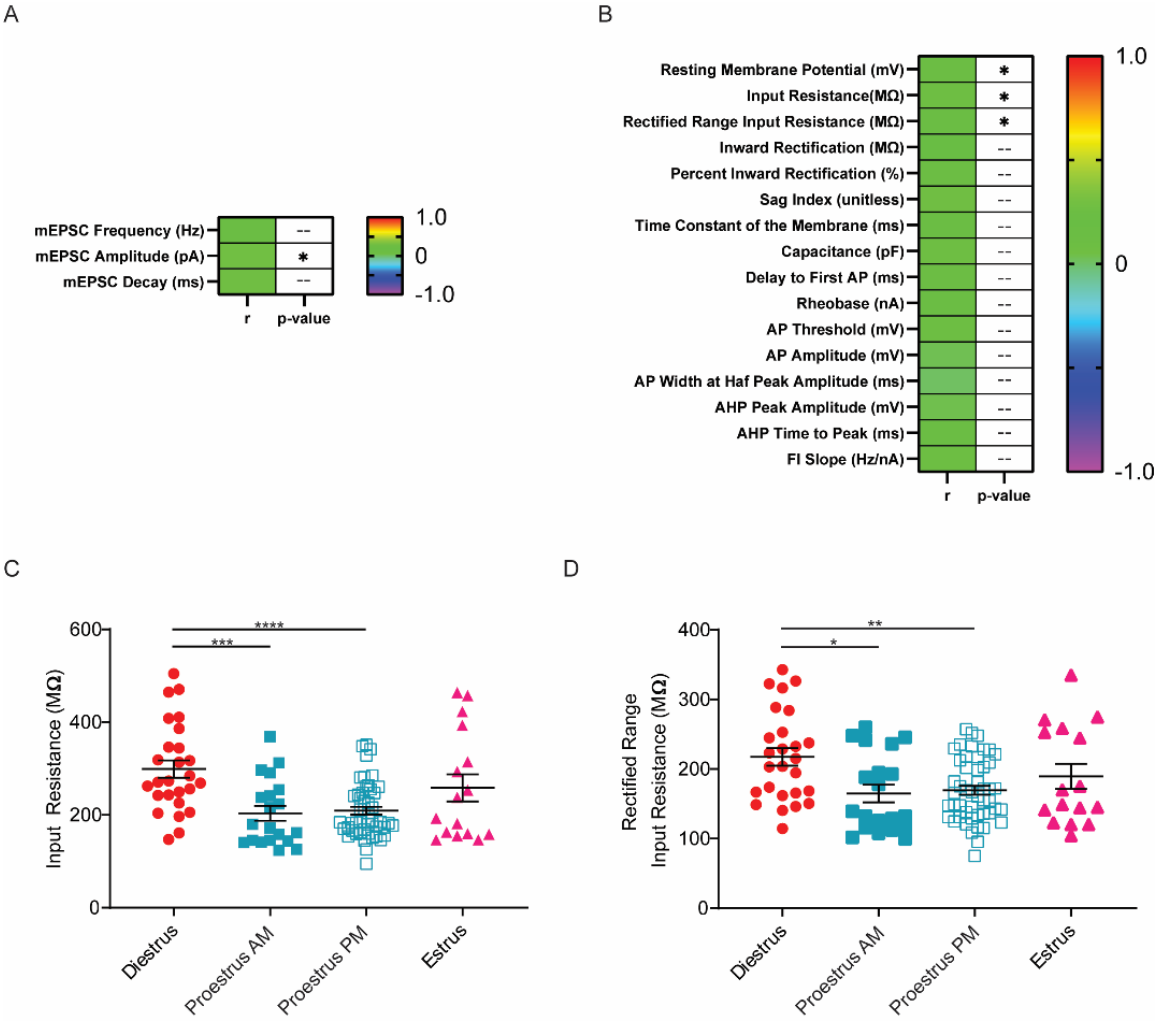

Figure 3.7. Correlation of electrophysiological properties across all phases of the estrous cycle with circulating levels of estradiol and progesterone. *A*. Correlation of miniature excitatory post synaptic current (mEPSC) properties with circulating levels of estradiol and progesterone. *B*. Correlation of intrinsic excitability properties with circulating levels of estradiol and progesterone. Statistically significant correlations in *A* and *B* are designated with *. *C* and *D*: input resistance in the linear range *(C)* and input resistance in the rectified range *(D)* vary across estrous cycle phase. Horizontal line superimposed upon scatterplots in *C* and *D* indicates the mean. Lines above the scatterplots indicate statistical significance. **P<0.05, **P<0.01, ****P<0.0001*.

Discussion

The findings presented here demonstrate differential and synergistic roles of estradiol and progesterone in the modulation of AcbC MSN electrophysiological properties across the estrous cycle. Circulating levels of estradiol correlate with resting membrane potential, the time constant

of the membrane, and rheobase, while circulating levels of progesterone correlate with mEPSC frequency and amplitude. Furthermore, when analyzed in combination, circulating levels of estradiol and progesterone correlate with mEPSC amplitude, resting membrane potential, and input resistance in both the linear and rectified ranges. These correlations compare with the changes in MSN properties across estrous cycle phases, including the novel proestrus PM phase presented in this study, which features a notable decrease in mEPSC frequency. Collectively, this study provides the first mechanistic clues of the underlying neuroendocrine mechanisms that shape AcbC input-output properties, which bears relevance to the associated changes in AcbC-mediated behaviors and functions across the estrous cycle.

Our previously published data on AcbC properties across the estrous cycle established two competing interpretations for the changes in MSN excitatory synapse and intrinsic excitability properties. One interpretation is that these changing properties directly facilitate changes in AcbC function with resulting impacts on behavior. A second interpretation suggests that changes in excitatory synaptic input and intrinsic excitability compensate for each other. This type of change would mitigate differential circuit output, perhaps via a homeostatic synaptic plasticity mechanism. This second interpretation is based upon potentially contrasting changes in excitatory synapse properties and intrinsic excitability between diestrus MSNs when compared to proestrus AM and estrus MSNs. Briefly, MSNs in the diestrus phase demonstrated decreased mEPSC frequency and increased intrinsic excitability compared to proestrus AM and estrus phase MSNs. This dataset, however, did not include metrics from AcbC MSNs in the proestrus PM phase. This omission was notable given that the proestrus PM phase of the estrous cycle demonstrates different hormonal, behavioral, and reproductive functions compared to other phases of the cycle. As demonstrated by the current study, the potentially contrasted changes in

excitatory synapse properties and intrinsic excitability exhibited between diestrus when compared to proestrus AM and estrus dissipated during proestrus PM (Figure 3.8).

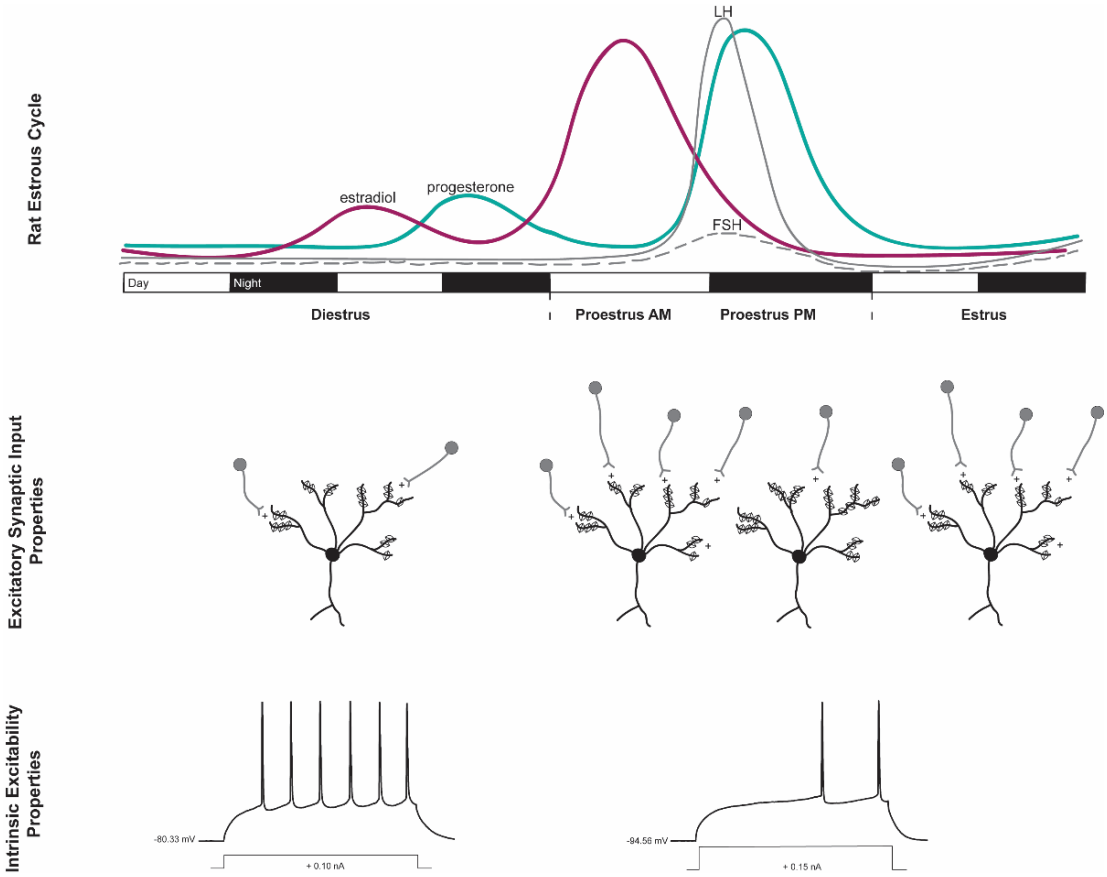

Figure 3.8. Schematic of AcbC MSN excitatory synapse and intrinsic excitability properties between estrous cycle phases in the adult female rat. Excitatory synapse properties are decreased in the diestrus stage of the cycle while intrinsic excitability is increased. In proestrus AM, excitatory synapse properties are elevated, and intrinsic excitability is decreased while in the proestrus PM excitatory synapse properties are drastically reduced and intrinsic excitability properties remain comparable to those in proestrus AM and estrus. In the estrus phase, excitatory synapses recover to levels seen in the proestrus AM while intrinsic excitability is decreased, and levels are comparable to those in proestrus AM and PM.

These findings indicate that these properties are dissociable, potentially controlled by different hormones, and are likely not a type of homeostatic plasticity. In short, changes in excitatory synaptic input can be disengaged from changes in intrinsic excitability, and vice versa. Another line of evidence arguing against homeostatic plasticity is the heterogeneity in electrophysiological changes and their relationship to circulating levels of either estradiol, progesterone, or the combination of both, as may occur during the estrus phase. Several

electrophysiological properties during the estrus phase assumed a more diestrus-like phenotype, others assumed a more proestrus-like (both AM and PM) phenotype, while others acquired more intermediate values. This suggests that specific cellular properties revert to a 'non-hormone influenced' state at different timescales, indicating temporal differentiation as well as divergent mechanism of hormone action, and further arguing against the presence of homeostatic synaptic plasticity. Thus, we believe that the preponderance of evidence favors the first interpretation: changing electrophysiological properties across the estrous cycle directly facilitate changes in AcbC function.

Intriguingly, circulating levels of estradiol alone correlate with intrinsic excitability but not excitatory synapse properties. Circulating levels of estradiol correlated with resting membrane potential, the time constant of the membrane, and rheobase, which all determine neuronal excitability. There are several estrogen receptor types that may be responsible for this action, as the AcbC and other striatal regions express membrane-associated estrogen receptors (ERs) α, β, and GPER-1 with sparse or no expression of nuclear receptors in adult animals (Almey et al 2012, Almey et al 2015). We do note that a detailed characterization of estrogen receptor expression across development and estrous cycle phase has not been published. A potential mechanism for these changes in membrane excitability properties is through estradiol's action on mERα, mERβ, along with their association with metabotropic glutamate receptors, to induce L-type calcium currents as well as CREB phosphorylation in the striatum (Grove-Strawser et al 2010, Mermelstein et al 1996). The lack of a correlation with estradiol and excitatory synapse properties is somewhat puzzling. One explanation for this is that the primary actions of estradiol on excitatory synapse properties occur after peak estradiol actions are reached, preventing a statistical correlation. Consistent with this explanation, previous studies

have found that multiple estradiol injections in female rats change AcbC-associated behaviors as well as dopaminergic and glutamatergic synaptic transmission (Becker & Rudick 1999, Cao et al 2018, Krentzel et al 2019, Krentzel et al 2020, Miller et al 2020). Important for excitatory synapse properties, an overall decrease in dendritic spine density was reported in AcbC but not caudate-putamen MSNs (Peterson et al 2015). In addition, sex differences in excitatory synapse number and associated markers in AcbC MSNs have been identified between proestrus females and males (Forlano & Woolley 2010, Wissman et al 2012). Inconsistent with this explanation, in adult female but not male AcbC, bath estradiol administration rapidly decreased mEPSC frequency (Krentzel et al 2019). However, one caveat to the study from Krentzel and colleagues is that it was not designed to detect potential differences across the estrous cycle. We also note that another caveat, for both Krentzel 2019 and the current study, is that MSN subtype was not addressed. It is possible that estradiol and progesterone differentially modulate MSNs depending on the type of dopamine receptor expressed, either through direct or indirect actions via afferent excitatory inputs. Importantly, aromatase may also be present in the striatum (Horvath et al 1997, Jakab et al 1993, Tozzi et al 2015, Wagner & Morrell 1996). Thus, it remains possible that both *de novo* synthesis of estradiol in the striatum and ovarian estradiol can regulate AcbC electrophysiological properties in both acute and longer-term time frames. Collectively, these data indicate that AcbC MSNs may exhibit a specific long-term estradiol sensitivity in intrinsic excitability and an acute or perhaps even longer-term estradiol sensitivity in excitatory synapse properties that could be related to the AcbC's role in motivated reproductive behaviors (Tonn Eisinger et al 2018) including, perhaps, rapid changes in sexual receptivity detected during the estrous cycle (Micevych et al 2017, Yoest et al 2018), paced mating behavior (Jenkins & Becker 2001), sexual reward (Meisel & Mullins 2006), and locomotor and anxiety-related behaviors

(Krentzel & Meitzen 2018, Krentzel et al 2020, Miller et al 2020). Importantly, correlation is not causation, and an important future direction of this research will be to establish causal roles of circulating estradiol and progesterone levels via exogenous hormone supplementation of ovariectomized females.

Though most striatal sex-focused research has concentrated on estradiol (Meitzen et al 2018, Yoest et al 2014), progesterone is also an important factor for the estrous cycle, especially for the proestrus PM and estrus phases. In this study, we found that progesterone correlated with several excitatory synapse properties, including mEPSC frequency and amplitude. Consistent with a possible relationship of progesterone and excitatory synapse properties, mEPSC frequency during proestrus PM drastically decreased to levels unrivaled by other estrous cycle phases while maintaining comparable levels of intrinsic excitability to proestrus AM and estrus MSNs. This sharp decrease in mEPSC frequency suggests a curtailment in excitatory synaptic properties that might be mediated by an acute effect of progesterone, in addition to the acute action of estradiol discussed above (Krentzel et al 2019). Regardless of the underlying mechanism, there is precedent for progesterone inducing differences in synapse properties either in the context of the estrous cycle and via exogenous supplementation in ovariectomized animals. Progesterone exposure has been shown to rapidly decrease dendritic spine density in pyramidal neurons in area CA1 of the hippocampus in female rats during the 24-hour window between proestrus PM and estrus (Woolley & McEwen 1993). A decrease in dendritic spine density often but not always correlates with a decrease in mEPSC frequency. Thus, it is possible that just as in the hippocampus, increasing circulating levels of progesterone in the AcbC during proestrus PM may induce the sharp decrease in mEPSC frequency, bearing in mind that this may be one of several mechanisms that synergistically work to decrease excitatory synapse properties.

Regarding specific progesterone action in the AcbC and other striatal regions, select studies have directly addressed this question. Importantly, evidence for progesterone receptor expression in the striatal regions has been documented in several species that include rodents and birds. Thus, here we will briefly review relevant evidence for striatal progesterone action. A primary culture study on striatal cells from C57B2/J6 mice detected progesterone receptor encoding mRNA (Piechota et al 2017). In rats, bovine serum albumin (BSA)-conjugated progesterone bound to striatal cells demonstrating the presence of membrane-associated progesterone receptors (Dluzen & Ramirez 1989b, Ke & Ramirez 1990). A study in the domestic hen identified AcbC cell nuclei expressing progesterone receptors using immunohistochemistry (Sterling et al 1987). In reference to progesterone action in the striatum, a study from 1984 found that progesterone exerted a biphasic effect on dopamine release. Progesterone administration to OVX-estradiol-primed female rats 2 to 12 hours before sacrifice potentiated spontaneous- and amphetamine-induced dopamine release while progesterone administration 24 hours before sacrifice largely inhibited the spontaneous- and amphetamine-induced release of dopamine (Dluzen & Ramirez 1984). This difference in temporal action of progesterone is highly suggestive of possible differential temporal actions in the context of the estrous cycle. Another study by the same group found that amphetamine application along with a pulsatile administration of BSA-conjugated progesterone produced maximal levels of dopamine release compared to continuous BSA-conjugated progesterone application or vehicle controls (Dluzen & Ramirez 1989a, Dluzen & Ramirez 1989b). In a different study, progesterone and NMDA application onto striatal slices from proestrus female rats amplified the release of dopamine that was normally caused by NMDA administration alone (Cabrera & Navarro 1996). These studies suggest a close interaction between progesterone and glutamatergic systems in the striatum that

modulate the release of dopamine, in addition to the well-known role of estradiol. In a pathological context, progesterone has been shown to modulate AcbC-mediated behaviors related to drugs of abuse (Becker 1999). Cocaine induced place preference behavior was decreased in rats that received an acute dose of progesterone (Russo et al 2003). In humans, women in the luteal phase of the menstrual cycle, when progesterone levels are high, have a reduced desire to smoke cocaine and an attenuated subjective response to cocaine than during the follicular phase (Evans & Foltin 2006, Sofuoglu et al 2002), albeit the luteal phase also features moderate levels of estradiol. Finally, in addition to a possible role of progesterone in regulating excitatory synapse, progesterone metabolites such as allopregnanolone have long been documented to act on GABA receptors, which AcbC MSNs also express (Bitran et al 1995). Collectively, this body of evidence suggests that progesterone may modulate the electrophysiology AcbC MSNs and resulting behaviors.

So far, we have discussed the effects of estradiol and progesterone separately. However, our study also indicates that some electrophysiological properties correlate with estradiol and progesterone in combination. These properties include mEPSC amplitude, resting membrane potential, and input resistance in both the linear and rectified ranges. The combined action of estradiol and progesterone in the regulation of these properties is supported by the dual action of these hormones in proestrus and potential after-actions in estrus. This suggests several possible mechanisms that may induce the sharp decrease in mEPSC frequency in proestrus PM (Figure 8). All of these possible mechanisms focus upon a presynaptic locus of action. This is because the changes in mEPSC frequency are much more robust than changes in mEPSC amplitude. With this written, we acknowledge that there are potential postsynaptic actions as well. Regarding presynaptic mechanisms, first, estradiol and progesterone may both decrease mEPSC

properties, but via potentially different mechanisms and timeframes. Following an acute decrease in AcbC MSN mEPSC frequency via estradiol (Krentzel et al 2019), progesterone may also acutely decrease mEPSC frequency, which is a testable hypothesis and can be disproved or confirmed by future experiments. Both hormones may also induce decreases in dendritic spine density, with estradiol and progesterone exposure speeding this decrease as has been show using exogenous exposure to ovariectomized rat hippocampus (McEwen & Woolley 1994, Woolley & McEwen 1993). If this model is accurate, then it predicts that dendritic spine density will be decreased in the AcbC during proestrus PM and potentially estrus. It also predicts that, like exogenous estradiol exposure, exogenous progesterone exposure likewise decreases dendritic spine density in AcbC MSNs. This model does not rule out other, synergistic potential mechanisms. One mechanism is a possible association between ovarian hormones and glial cells across the estrous cycle. Data indicate that the number of axosomatic excitatory synapses on neurons in the arcuate nucleus of the hypothalamus begins to decrease during proestrus AM, remains low until the morning of estrus, and then rises again by diestrus (Naftolin et al 2007, Olmos et al 1989). This change in excitatory synapse number is not because of a disappearance of excitatory synapses *per se* but is rather due to remodeling of astroglia and astroglial processes (Garcia-Segura et al 1994, Kohama et al 1995). This idea of fast changes in excitatory synapse properties is not limited to the arcuate nucleus, as a similar but not identical process has also been detected in the anteroventral periventricular nucleus (Langub et al 1994). If this process is present in the AcbC, then differences in glia and perhaps microglia function should be detectable across the estrous cycle phases. Evidence of microglia sex-specifically altering dopamine inputs in the AcbC during development and puberty has already been presented (Kopec et al 2018). Further studies could shed light on each of these potential mechanisms. Considered together, a

comprehensive action of direct and combinatorial estradiol and progesterone action, a decrease in dendritic spines, and glial ensheathment may collectively act to decrease excitatory synaptic input onto AcbC MSNs during proestrus PM, potentially inducing the profound alteration of AcbC MSN excitatory synapse properties detected during proestrus PM.

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CHAPTER 4

Estradiol decreases medium spiny neuron excitability in female rat nucleus accumbens core

Research in this chapter is under review with the following journal:

Proaño, S.B. and Meitzen, J. (2020). Estradiol decreases medium spiny neuron excitability in female rat nucleus accumbens core. *Journal of Neurophysiology*.

Abstract

The menstrual cycle in humans and its analogous cycle in rodents, the estrous cycle, modulate brain function and behavior. Both cycles are characterized by the cyclical fluctuation of ovarian hormones, including estrogens such as estradiol. Estradiol induces cycle- and sex-dependent differences in the phenotype and incidence of many behaviors, including those related to reward and motivation. The nucleus accumbens core (AcbC), a limbic and premotor system nexus region, directly regulates these behaviors. We previously showed that the estrous cycle modulates intrinsic excitability and excitatory synapse properties of medium spiny neurons (MSNs) in the AcbC. The identity of the underlying hormone mechanism is unknown, with estradiol being a prime candidate. The present study tests the hypothesis that estradiol induces estrous cycle relevant differences in MSN electrophysiology. To accomplish this goal, a time- and dose-dependent estradiol replacement paradigm designed to simulate the rise of circulating estradiol levels across the estrous cycle was employed in ovariectomized adult female rats, as well as a vehicle control group. Estradiol replacement decreased MSN excitability by modulating properties such as resting membrane potential, input resistance in both the linear and rectified ranges, and rheobase, compared to vehicle treated females. These differences in MSN excitability mimic those previously described regarding estrous cycle effects on MSN electrophysiology. Excitatory synapse properties were not modulated in response to this estradiol replacement paradigm. These data are the first to demonstrate that an estrous cycle relevant estradiol exposure modulates MSN electrophysiology, providing evidence of the fundamental neuroendocrine mechanisms regulating the AcbC.

Introduction

Female humans and rodents of reproductive age exhibit cyclical fluctuations of ovarian sex steroid hormones including estrogens, the focus of this study. In humans, cyclical changes in the production of ovarian estrogens such as estradiol occur over the ~28-day menstrual cycle in which circulating levels of estradiol peak during the follicular phase (Sherman & Korenman 1975). In rodents, including rats, fluctuations in ovarian hormones occur over the ~4-5-day estrous cycle. In rats, circulating levels of estradiol gradually rise during the diestrus phase, reach peak levels during the proestrus phase, and then quickly decrease, with effects remaining through the estrus phase (Erskine 1989, Hubscher et al 2005, Westwood 2008). Accumulating evidence suggests that, in both humans and rodents, fluctuations in ovarian estrogen production influence behaviors related to motivation, reward and reinforcement, and related disorders such as anxiety, depression, and addiction (Becker & Hu 2008, Becker et al 2001, Evans & Foltin 2006, Lebron-Milad & Milad 2012, Milad et al 2009, Tonn Eisinger et al 2018a, Yoest et al 2018). The underlying neural substrates mediating these behaviors are likewise susceptible to ovarian estrogen fluctuations. Indeed, neural physiology in the striatal regions implicated in these behaviors and disorders, including the caudate-putamen and nucleus accumbens core (AcbC), differs across the estrous cycle (Alonso-Caraballo & Ferrario 2019, Proano et al 2018, Willett et al 2019b). Previously, we showed that the estrous cycle robustly modulates the intrinsic excitability and excitatory synapse properties of female rat medium spiny neurons (MSNs) in the AcbC (Proano et al 2018), a critical nexus region between the limbic and premotor systems that mediates the cognitive processing of reward and reinforcement, among other functions (Floresco 2015, Francis & Lobo 2017). These physiological findings support the differential expression of

AcbC-mediated behaviors and disorders across the estrous cycle in rodents and the menstrual cycle in humans.

Accumulating evidence has firmly established estradiol as a potent modulator of AcbC function (Meitzen et al 2018, Yoest et al 2018). The output neurons of the AcbC, medium spiny neurons (MSNs), in adulthood express membrane-associated estrogen receptors α, β, and GPER-1 (Almey et al 2015, Lorsch et al 2018). MSNs integrate a host of inputs, including dopamine from the ventral tegmental area and glutamatergic and GABAergic projections from cortex, amygdala, thalamus and hippocampus (Deroche et al 2020, Kalivas & Nakamura 1999, Salgado & Kaplitt 2015). These neurotransmitters are sensitive to estradiol in a sex-, temporal- and developmental stage-dependent manner (Becker 1999, Becker et al 2012, Calipari et al 2017, Cao et al 2016, Cao et al 2018a, Forlano & Woolley 2010, Krentzel et al 2019, Krentzel et al 2020, Meisel & Mullins 2006, Mermelstein et al 1996, Perry et al 2013, Tonn Eisinger et al 2018a). Estradiol also modifies MSN transcription factor phosphorylation and dendritic spine structure by activating metabotropic glutamate receptor 5 (mGluR5) signaling (Grove-Strawser et al 2010, Mermelstein et al 1996, Peterson et al 2015, Staffend et al 2011). However, estradiol's actions on the electrophysiological properties of MSNs, especially within the context of a natural variable such as the estrous cycle, are not well defined. This is a critical knowledge gap given the aforementioned differential expression of MSN electrophysiological properties across the estrous cycle (Proano et al 2018) which along with changes in neurotransmitter systems, could potentially underlie the sex- and estrous-cycle reported differences in AcbC-mediated behaviors and disorders. In the present study, we address this gap by testing the hypothesis that estradiol injections designed to simulate circulating estradiol levels during the estrous cycle modulates AcbC MSN electrophysiological properties. To accomplish this, we

administered sequential doses of estradiol or vehicle to ovariectomized (OVX) female rats and then performed whole-cell patch clamp recordings of AcbC MSNs to assess both intrinsic and excitatory synapse properties.

Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University. Female Sprague-Dawley CD IGS rats were born from timed-pregnant dams purchased from Charles River Laboratories. Rats were housed with their littermates and dam until weaning at postnatal day (P)21. After weaning, females were group housed until they were bilaterally gonadectomized at P60±2 as previously described (Krentzel et al 2020). Upon gonadectomy, animals were individually housed to facilitate estradiol injection administration which began at P74±2: vehicle control (n=11) and estradiol (n=15). Injection paradigm is documented below (Figure 4.1). Age at recording ranged from P73 to P85 and was matched between the groups (mean ± SE; vehicle control: 78 ± 1 and estradiol injected: 79 ± 1). All animals were housed in a temperature- and light-controlled room (23 °C, 40% humidity, 12:12-h light-dark cycle) at the Biological Resource Facility of North Carolina State University. All cages were washed with polysulfone Bisphenol A (BPA) free and were filled with bedding manufactured from virgin hardwood chips (Beta Chip; NEPCO, Warrensburg, NY) to avoid the presence of endocrine disruptors in corncob bedding (Mani et al 2005, Markaverich et al 2002, Villalon Landeros et al 2012). Soy protein-free rodent chow (2020X; Teklad, Madison, WI) and glass bottle-provided water were available *ad libitum*.

Estradiol injection protocol

One week after gonadectomy, female rats were handled (picked up, cradled, and stroked) twice daily until day of sacrifice. Two weeks after gonadectomy, animals experienced a two-day injection acclimatization period via injections of 0.3 mL of normal saline (Sigma) twice daily at 9:30 AM and 9:30 PM. Estradiol or vehicle injections began after the two-day injection acclimatization period. Estradiol injection protocols were from a previously published study (Scharfman et al 2007) and mimic the changing temporal circulating levels of estradiol during the diestrus and proestrus phases of the rat estrous cycle. Stock solutions of estradiol benzoate (EB) and 17-β estradiol (E_2) (collectively referred as estradiol in this manuscript) were made by diluting a concentrated stock solution (1 mg of EB or E_2 in 1 mL of DMSO) in corn oil (Sigma). Stocks were stored in the dark at room temperature. Injection volume (0.3 mL) was kept constant by using stock solutions that contained differing concentrations of EB and E_2 (3 µg/mL for a 3 µg/kg dose and 4 µg/mL for a 4µg/kg dose). Vehicle solutions were prepared in the same manner using DMSO and corn oil. Injections were administered subcutaneously in the back using a 1 mL syringe and a 26-gauge needle. 3 µg/mL of EB or vehicle were injected at 9:30 AM of day 1, 4 µg/mL of EB or vehicle were injected at 9:30 PM on day 1, and 3 µg/mL of E_2 or vehicle were injected at 9:30 AM of day 2. Two hours (11:30 AM) after the last injection, animals were processed for acute brain slice preparation (Figure 4.1).

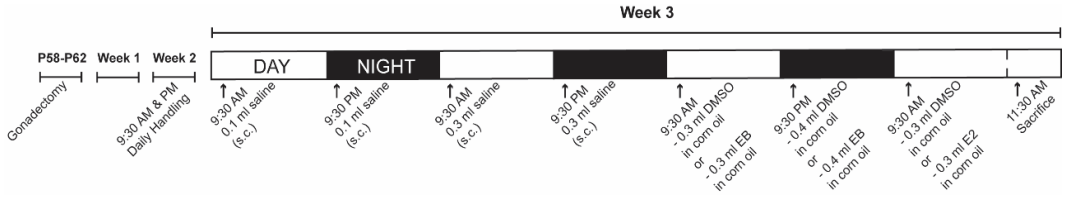

Figure 4.1. Estradiol replacement protocol. Two weeks after gonadectomy, female rats received a series of subcutaneous injections of estradiol benzoate and 17-β estradiol to simulate temporal changes in physiologically relevant circulating levels of estradiol during the estrous cycle. Animals were sacrificed during the replacement protocol equivalent of the proestrus phase. Protocol was originally developed and validated by Scharfman and colleagues, 2007.

Acute brain slice preparation

Brain slices containing the nucleus accumbens core were prepared as previously described (Proano et al 2018). Animals were deeply anesthetized with isoflurane gas before decapitation. The brain was rapidly extracted into ice-cold oxygenated sucrose artificial cerebrospinal fluid containing (in mM) 75 sucrose, 1.25 NaH_2PO_4, 3 $MgCl_2$, 0.5 $CaCl_2$, 2.4 Na pyruvate, and 1.3 ascorbic acid from Sigma-Aldrich (St. Louis, MO) and 75 NaCl, 25 $NaHCO_3$, 15 dextrose, and 2 KCl from Fisher (Pittsburgh, PA). The osmolarity of the sucrose ACSF was 295–305 mosM, and the pH was between 7.2 and 7.4. Coronal brain slices (300 m) were prepared with a vibratome and then incubated in regular ACSF containing (in mM) 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, and 2 $CaCl_2$ (295–305 mosM, pH 7.2–7.4) for 30 min at 30–35°C and then for at least 30 min at room temperature (22–23°C). Slices were stored submerged in room-temperature oxygenated ACSF for up to 6 h after sectioning in a large-volume bath holder.

Electrophysiological recordings

Slices rested for at least 1 h after sectioning. They were then placed in a Zeiss Axioscope equipped with IR-DIC optics, a Dage IR-1000 video camera, and 10x and 40x lenses with optical zoom and superfused with oxygenated ACSF heated to ~29.0°C. Whole cell patch-clamp recordings were used to record the electrical properties of MSNs in the nucleus accumbens core (Figure 4.2). Glass electrodes contained (in mM): 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 $MgCl_2$, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine from Sigma-Aldrich and 10 HEPES from Fisher (285 mosM, pH 7.2–7.4). Signals were amplified, filtered (2 kHz), and digitized (10 kHz) with a MultiClamp 700B amplifier attached to a Digidata 1550 system and a personal computer using pCLAMP 10.7 software. Membrane potentials were corrected for a calculated liquid junction potential of 13.5 mV. As previously described (Dorris et al 2015), recordings

were first made in current clamp to assess neuronal electrophysiological properties. MSNs were identified by medium-sized somas, the presence of a slow-ramping subthreshold depolarization in response to low-magnitude positive current injections, a hyperpolarized resting potential more negative than -65 mV, inward rectification, and prominent spike afterhyperpolarization (Belleau & Warren 2000, O'Donnell & Grace 1993). In a subset of recordings, oxygenated ACSF containing both the $GABA_A$ receptor antagonist picrotoxin (PTX, 150 M; Fisher) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 M; Abcam Biochemicals) was applied to the bath to abolish inhibitory postsynaptic current events and action potentials, respectively. Once depolarizing current injection no longer generated an action potential, MSNs were voltage clamped at -70 mV and mEPSCs were recorded for at least 5 min. These settings enable recordings from almost exclusively AMPA glutamate receptors (Nowak et al 1984) and was confirmed by our laboratory in a previous study (Proano et al 2018). Input/series resistance was monitored per minute, and cells were excluded if resistance changed more than 25%.

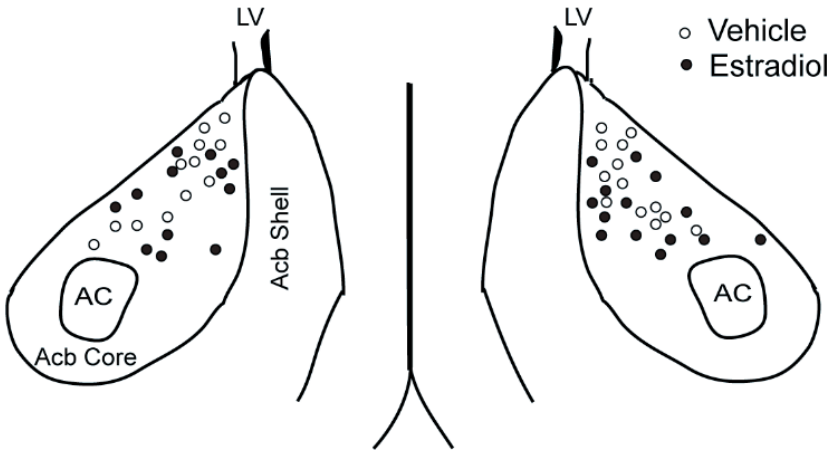

Figure 4.2. Location of AcbC whole-cell patch clamped MSNs from estradiol- and vehicle-treated female rats. AC, anterior commissure; LV, lateral ventricle.

Data recording and analysis

Intrinsic electrophysiological properties and action potential characteristics were analyzed with pCLAMP 10.7. After break-in, the resting membrane potential was first allowed to stabilize ~1–2 min, as previously described (Mu et al 2010). After stabilization, resting membrane potential was assessed in the absence of injected current. At least two series of depolarizing and hyperpolarizing current injections were applied to elicit basic neurophysiological properties. All properties measured followed definitions previously adopted by our laboratory (Cao et al 2016, Dorris et al 2015, Proano et al 2018, Willett et al 2019b, Willett et al 2016) which were based on those of Perkel and colleagues (Farries et al 2005, Farries & Perkel 2000, Farries & Perkel 2002, Meitzen et al 2009). For each neuron, measurements were made of at least two action potentials generated from minimal current injections. These measurements were then averaged to generate the reported action potential measurement for that neuron. For action potential measurements, only the first generated action potential was used unless more action potentials were required to meet the standard three action potentials per neuron. Action potential threshold was defined as the first point of sustained positive acceleration of voltage ($\delta^2V/\delta t^2$) that was also 3 times the SD of membrane noise before the detected threshold (Baufreton et al 2005). The delay to first action potential is the average time in milliseconds of the time from the initial deflection generated by the current step function to the action potential threshold of the first spike. Action potential width at half peak is the width of the action potential halfway between action potential peak and threshold in milliseconds. The action potential amplitude is the change in millivolts between action potential threshold and peak. Afterhyperpolarization peak amplitude is the difference in millivolts between action potential threshold and the most hyperpolarized voltage point after action potential peak.

Afterhyperpolarization time to peak amplitude is the time measured in milliseconds between the action potential threshold voltage point on the descending phase of the action potential and the afterhyperpolarization peak amplitude. Rheobase, measured in nanoamps, is the lowest amplitude of injected positive current needed to produce an initial action potential. The slope of the linear range of the evoked action potential firing rate-to-positive injected current curve (FI slope) was calculated from the first current stimulus that evoked an action potential to the first current stimulus that generated an evoked firing rate that persisted for at least two consecutive current stimuli. Input resistance in the linear, non-rectified, range was calculated from the steady-state membrane potential in response to 0.02-nA hyperpolarizing pulses. Rectified range input resistance, inward rectification, and percent inward rectification were calculated as described previously, with rectified range input resistance measured using the most hyperpolarizing current injected into the MSN (Belleau & Warren 2000). Inward rectification is the input resistance of the 0.02-nA step minus the rectified range input resistance. Percent inward rectification is defined as rectified range input resistance/input resistance x 100. The time constant of the membrane was calculated by fitting a single exponential curve to the membrane potential change in response to 0.02-nA hyperpolarizing pulses. Possible differences in hyperpolarization induced "sag" were assessed with the "sag index" (Farries et al 2005). Briefly, the sag index is defined as the difference between the minimum voltage measured during the largest hyperpolarizing current pulse and the steady-state voltage deflection of that pulse, divided by the steady-state voltage deflection. A cell with no sag would exhibit a sag index of 0, whereas a cell whose maximum voltage deflection is twice that of the steady-state deflection would exhibit a sag index of 1. Cells with considerable sag typically have an index of 0.1. Frequency, amplitude, and decay of mEPSCs were analyzed off-line with Mini Analysis (Synaptosoft,

http://www.synaptosoft.com/MiniAnalysis/). mEPSC threshold was set at a minimum value of 5 pA, and accurate event detection was validated by visual inspection. mEPSC frequency was defined as the number of detected mEPSC events per second (Hz). mEPSC amplitude was calculated as the difference between the averaged baseline 10 ms before initial mEPSC rise and peak amplitude. mEPSC decay was calculated as the time required for peak mEPSC amplitude to return to baseline.

Statistics

Data were analyzed as appropriate with two-tailed *t* tests and Mann-Whitney *U* tests, as determined by a D'Agostino & Pearson normality test, or with linear regressions and ANCOVAs (GraphPad Prism 8). P values < 0.05 were considered *a priori* as significant and P values < 0.10 as a trend. Data are presented as means ± SE.

Results

Estradiol increases action potential rheobase and hyperpolarizes resting membrane potential

To test the hypothesis that action potential generation properties are influenced by estradiol, we injected a series of positive current injections into MSNs from estradiol- and vehicle-treated animals and analyzed the initiation and number of evoked action potentials (Figure 4.3A). Complete statistical information as well as documentation of all assessed electrophysiological properties is in Table 4.1. Regarding action potential initiation, the minimum amount of depolarizing current necessary to initiate an action potential significantly differed between estradiol- and vehicle-treated groups (Figure 4.3B). Rheobase was increased in MSNs recorded from estradiol-treated animals compared to vehicle-treated animals (Figure 4.3C). Changes in rheobase are usually complimentary to changes in action potential threshold, resting membrane potential, and/or input resistance. There were no changes in action potential

threshold (Table 4.1). Resting membrane potential was hyperpolarized in MSNs recorded from estradiol-treated animals (Figure 4.3D). This hyperpolarization is consistent with an increased rheobase value. This finding suggests that more excitatory current is required for action potential initiation in MSNs from estradiol-treated females. Overall, these results indicate that estradiol decreases excitability in adult female AcbC MSNs.

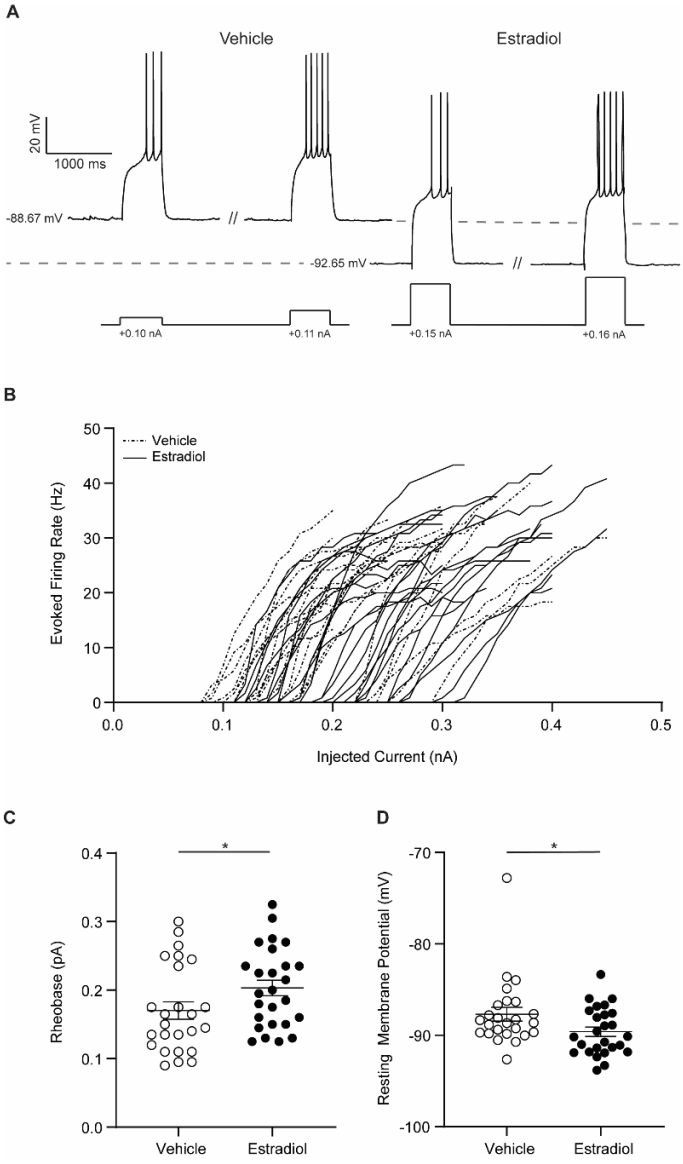

Figure 4.3. Action potential properties in MSNs recorded from estradiol- and vehicle-treated female rats. *A*. Voltage response of MSNs recorded from estradiol- and vehicle-treated animals to a series of depolarizing current injections. // denotes x axis breaks. *B*. Action potential firing rates evoked by depolarizing current injections. *C*. Rheobase. *D*. Resting membrane potential. Horizontal line superimposed upon scatterplots in *C* and *D* indicates the mean. Complete statistics are described in Table 4.1.

Estradiol decreases input resistance in both the linear and rectified ranges

The increase in rheobase in MSNs recorded from estradiol-treated animals may also be driven by a decrease in input resistance. To investigate input resistance in the linear and rectified ranges, we injected a series of hyperpolarizing current injections in MSNs from estradiol- and vehicle-treated animals (Figure 4.4A). The steady-state voltage deflection evoked by injected hyperpolarizing current curve (IV curve) (Figure 4.4B) showed that MSNs recorded from estradiol-treated females displayed a decreased voltage deflection in response to higher-magnitude hyperpolarizing current injections compared to MSNs recorded from vehicle-treated females. A linear regression analysis revealed that the slopes of the steady-state voltage deflections evoked by injected hyperpolarizing current curves between estradiol- and vehicle-treated animals are significantly different ($P<0.0001$). We further evaluated this difference by quantifying input resistance in both the linear and rectified ranges. Input resistance in the linear range is decreased in MSNs from estradiol-treated animals compared to MSNs from vehicle-treated animals (Figure 4.4C). Input resistance in the rectified range was also decreased in MSNs from estradiol-treated animals (Figure 4.4D). We also assessed whether this difference in the rectified range was due to an estradiol action on MSN inward rectification itself. No differences were detected in inward rectification or % inward rectification (Table 4.1), indicating that the difference in rectified-range input resistance is not due to changes in rectification per se. The estradiol-induced decrease in input resistance in both the linear and rectified ranges is consistent with the estradiol-induced increase in rheobase, indicating decreased excitability in MSNs recorded from estradiol-treated females.

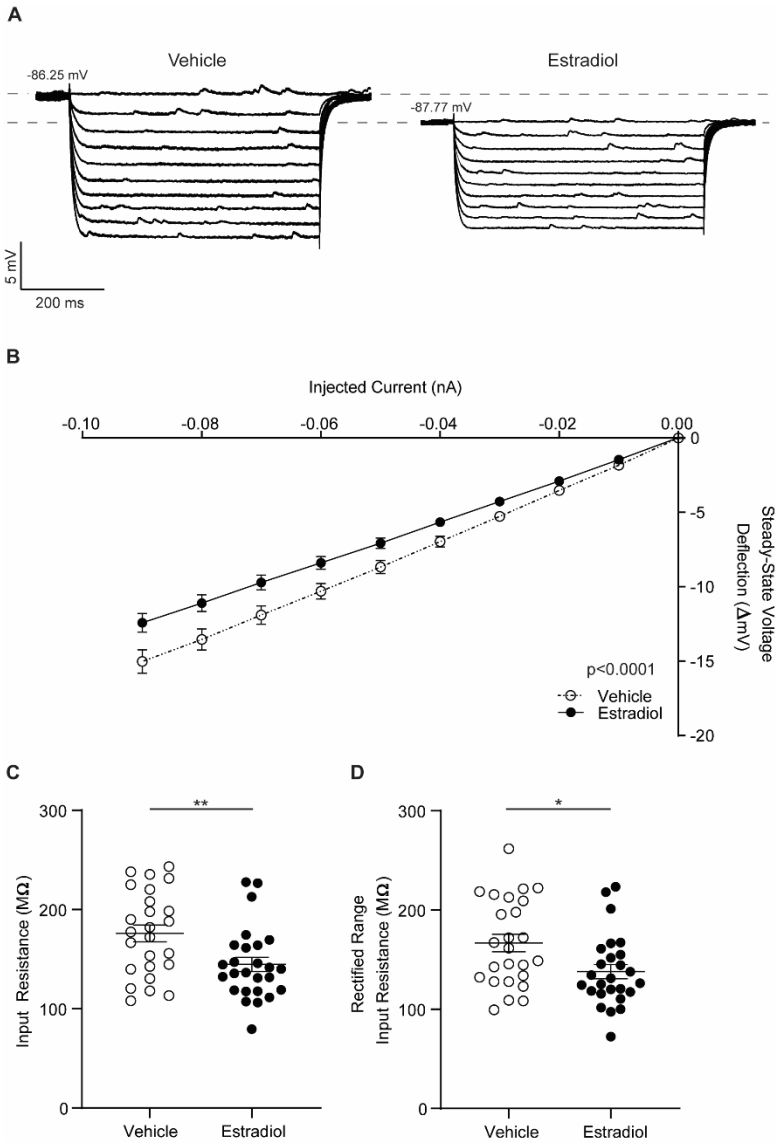

Figure 4.4. Input resistance properties from MSNs recorded from estradiol- and vehicle-treated female rats. *A*. Voltage response of MSNs recorded from estradiol- and vehicle-treated animals to a series of hyperpolarizing current injections. *B*. The injected current-to-steady-stage voltage deflection curve (IV curve). *C*. Input resistance in the linear range. *D*. Input resistance in the rectified range. Horizontal line superimposed upon scatterplots in *C* and *D* indicates the mean. Complete statistics are described in Table 4.1.

Individual action potential properties do not differ between estradiol- and vehicle-treated groups

We also tested whether individual action potential properties differed between estradiol- and vehicle-treated groups. MSNs from estradiol treated females trended toward a longer delay to first action potential in comparison to MSNs from vehicle-treated animals (Figure 4.5A; Table 4.1; p=0.084). This delay is a canonical aspect of MSNs and reflects the underlying slow

inactivating A current (Nisenbaum et al 1994). No differences were detected in action potential threshold (Figure 4.5B), action potential width at half-peak amplitude (Figure 4.5C), action potential amplitude (Figure 4.5D), action potential afterhyperpolarization time to peak amplitude (Figure 4.5E), or action potential afterhyperpolarization peak amplitude (Figure 4.5F). These findings indicate that individual action potential properties do not robustly differ between MSNs recorded from estradiol- and vehicle-treated animals.

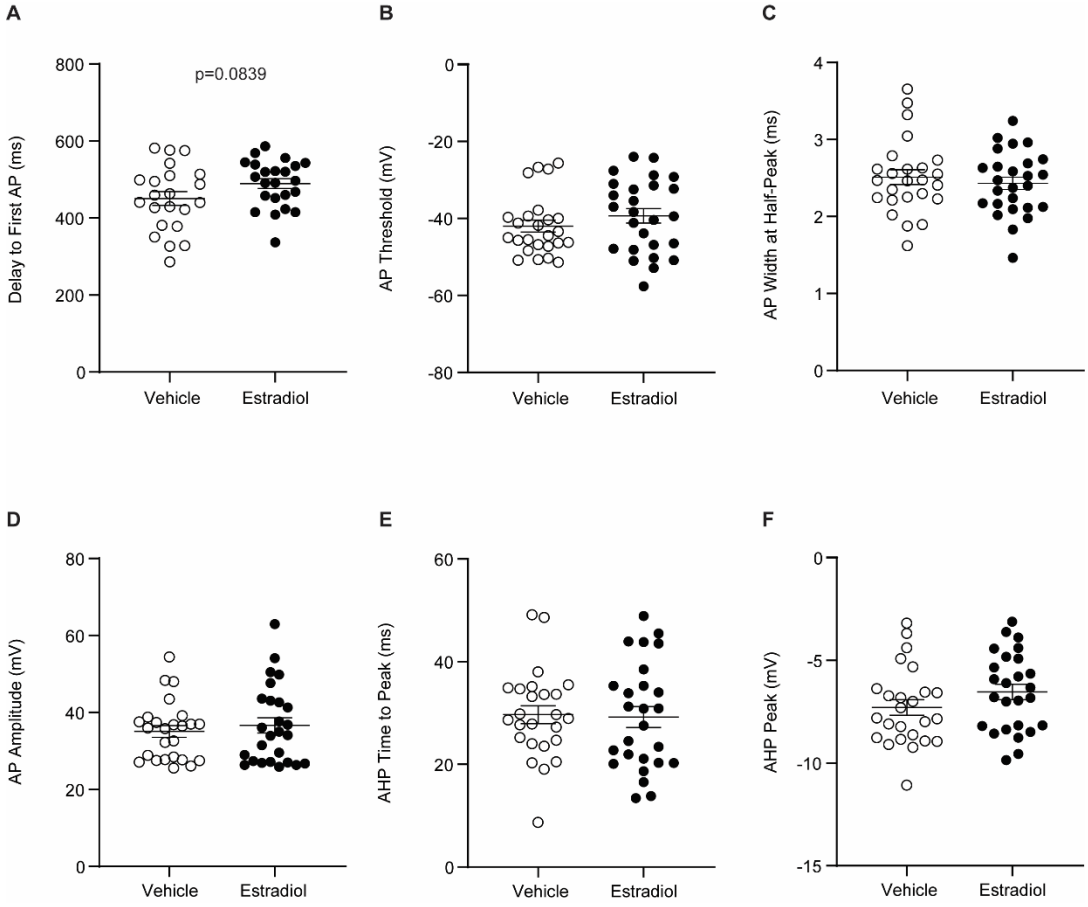

Figure 4.5. Individual action potential properties of MSNs recorded from estradiol- and vehicle-treated female rats. *A*. Delay to first action potential seemed longer in MSNs recorded from estradiol-treated MSNs but remained a statistical trend. *B*-*F*: action potential threshold (*B*), action potential width measured at half-peak amplitude (*C*), action potential amplitude (*D*), action potential afterhyperpolarization time to peak amplitude (*E*), and action potential afterhyperpolarization peak amplitude (*F*) did not differ between MSNs recorded from estradiol- and vehicle-treated animals. Horizontal line superimposed upon scatterplots in *A*-*F* indicates the mean. Complete statistics are described in Table 4.1.

mEPSC properties do not differ between estradiol- and vehicle-treated groups

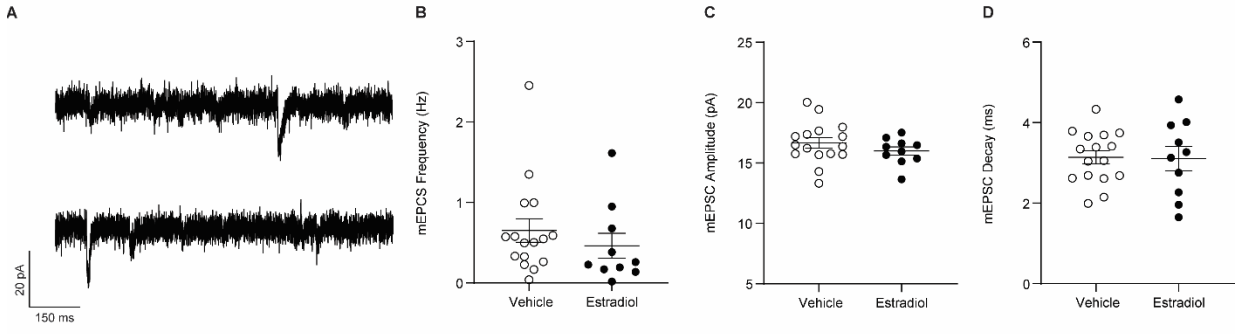

Figure 4.6. Miniature excitatory postsynaptic current (mEPSC) properties of MSNs recorded from estradiol- and vehicle-treated female rats. *A*. Representative examples of mEPSCs. *B*. mEPSC frequency. *C*. mEPSC amplitude. *D*. mEPSC decay. Horizontal line superimposed upon scatterplots in *B-D* indicates the mean. Complete statistics are described in Table 4.1.

mEPSC properties differ between estrous cycle phases in adult female rat AcbC (Proano et al 2018). To test whether this estradiol replacement paradigm influenced mEPSC properties, we voltage clamped MSNs from estradiol- and vehicle-treated animals to -70 mV and recorded mEPSCs while exposing MSNs to 1 μM TTX and 150 μM PTX to block sodium dependent action potentials and $GABA_A$ receptors, respectively (Figure 4.6A). We assessed mEPSC frequency, amplitude, and decay. There were no differences in mEPSC frequency (Figure 4.6B), mEPSC amplitude (Figure 4.6C), or mEPSC decay (Figure 4.6D). These findings indicate that mEPSC properties do not differ between MSNs recorded from estradiol- and vehicle-treated animals.

Table 4.1. AcbC MSN electrophysiological properties.

Property	**Vehicle**	**Estradiol**	**Statistics (*U/t, P*)**
mEPSC frequency (Hz)	0.7±0.1 (16)	0.5±0.2 (10)	55.500, 0.205
mEPSC amplitude (pA)	16.7±0.4 (16)	16.0±0.4 (10)	1.107, 0.279
mEPSC decay (ms)	3.1±0.2 (16)	3.1±0.3 (10)	0.101, 0.920
Resting membrane potential (mV)	**-87.7±0.8 (25)**	**-89.6±0.5 (26)**	**216, 0.040**
Input resistance (MΩ)	**176.1±8.5 (25)**	**144.8±7.1 (26)**	**185, 0.008**
Rectified range input resistance (MΩ)	**167.0±8.8 (25)**	**138.1±7.1 (26)**	**2.574, 0.013**
Inward rectification (MΩ)	9.1±2.7 (25)	6.8±1.1 (26)	0.819, 0.417
% inward rectification (%)	94.7±1.4 (25)	95.1±0.8 (26)	294, 0.569
Sag index (unitless)	-0.006±0.002 (25)	-0.000±0.003 (26)	1.412, 0.164
Time constant of the membrane (ms)	9.2±0.9 (25)	8.2±0.5 (26)	292, 0.544
Capacitance (pF)	55.5±6.9 (25)	59.5±4.1 (26)	250, 0.162
Rheobase (pA)	**0.17±0.01 (25)**	**0.20±0.01 (26)**	**220.5, 0.049**
Delay to first AP (ms)	*450.7±18.0 (22)*	*489.5±12.8(23)*	*1.770, 0.084*
AP Threshold (mV)	-42.0±1.5 (25)	-39.3±1.9 (26)	276, 0.364
AP amplitude (mV)	35.1±1.5 (25)	36.7±2.0 (26)	323, 0.978
AP width at half-peak amplitude (ms)	2.5±0.1 (25)	2.4±0.1 (26)	0.653, 0.517
AHP peak amplitude (mV)	-7.3±0.4 (25)	-6.5±0.4 (26)	1.419, 0.162
AHP time to peak (ms)	29.7±1.7 (25)	29.2±2.0 (26)	0.172, 0.864
FI Slope (Hz/nA)	298.1±15.0 (25)	304.5±11.9 (26)	283, 0.435

Excitatory synapse and intrinsic excitability properties recorded from AcbC MSNs from adult female rats treated with either vehicle or estradiol. Values are mean ± SEM. Numbers in parentheses indicate experimental "n". Bold font and colored cells indicate statistical significance. Italics indicate a statistical trend. AP, action potential; AHP, afterhyperpolarization; FI, evoked firing rate-to-positive current curve; mEPSC, miniature excitatory postsynaptic current.

Discussion

The findings presented here demonstrate that AcbC MSN membrane excitability is decreased by estrous cycle relevant doses of estradiol in female rats. Specifically, estradiol induced an increase in rheobase while concomitantly hyperpolarizing resting membrane potential and decreasing input resistance in both the linear and rectified ranges. The collective impact of these changes is to profoundly decrease overall MSN excitability. These findings recapitulated the changes observed in MSN excitability observed during the appropriate phases of the estrous cycle that likewise feature high estradiol levels or impact: proestrus and estrus, respectively

(Proano et al 2018). Together, these findings are the first to demonstrate a causal role for estrous-cycle relevant doses of estradiol in regulating AcbC MSN excitability.

This newly demonstrated causal link between estradiol and MSN electrophysiology builds on a line of research dating back to at least the early 1980s, if discussion is focused specifically on MSN electrophysiological properties. *In vivo* spontaneous firing rates and dopamine sensitivity of caudate-putamen MSNs changed when ovariectomized (OVX) female rats were administered estradiol or when ovary intact females were in the high estradiol impact phases of the estrous cycle (Arnauld et al 1981, Tansey et al 1983). Later, Mermelstein and colleagues demonstrated that estradiol rapidly decreased L-type calcium currents in dissociated female but not male rat caudate-putamen MSNs (Mermelstein et al 1996). This specific finding directly foreshadows the current work, although decreases in L-type calcium channel currents are more typically associated with intracellular signaling rather than resting membrane potential hyperpolarization. The first indication of a sex difference in nucleus accumbens MSN electrophysiological properties came from Wissman and colleagues, who showed that mEPSC frequency was increased in gonad intact female compared to male rats (Wissman et al., 2011). Our laboratory then built upon these findings by showing that rat MSNs in both the AcbC and caudate-putamen exhibit developmental- and estrous cycle-dependent sex differences in intrinsic excitability and excitatory synapse properties (Cao et al 2016, Cao et al 2018b, Dorris et al 2015, Proano et al 2018, Willett et al 2019b). Mice MSNs in general exhibit less robust sex differences than rat MSNs in both the AcbC and caudate-putamen (Cao et al 2018a, Willett et al 2019a). Regarding the rat AcbC, during the prepubertal period female rat MSNs exhibit increased mEPSC frequency but no differences in intrinsic excitability compared to male MSNs (Cao et al 2016). This increased mEPSC frequency in female prepubertal MSNs is abolished by

masculinizing/feminizing doses of estradiol or testosterone during the perinatal critical period. In adult gonad intact female rats, AcbC MSNs show changes in both membrane excitability and excitatory synapse properties across estrous cycle phases, which generates sex differences when compared to male MSNs. Regarding membrane excitability, MSNs recorded in proestrus AM and estrus showed hyperpolarized resting membrane potentials accompanied by decreases in input resistance and increases in rheobase (Proano et al 2018). These electrophysiological differences are abolished when females are ovariectomized. The current study employed estradiol doses that mimicked circulating estradiol levels experienced during the estrous cycle, and featured MSNs recorded during the equivalent of proestrus. This manipulation induced differences in membrane excitability that match what was previously observed in MSNs recorded from females in proestrus and estrus phases. As in proestrus, the MSNs recorded from estradiol-exposed females from the current study exhibited hyperpolarized resting membrane potential, decreased input resistance in both the linear and rectified ranges, and increased rheobase, indicating an overall decrease in membrane excitability.

Surprisingly, estrous cycle-relevant doses of estradiol did not induce differences in mEPSC excitatory synapse properties. This finding does not mimic the previous study of estrous cycling females, given that excitatory synapse properties such as mEPSC frequency, amplitude, and decay robustly differed across the estrous cycle. Specifically, mEPSC frequency is significantly increased during proestrus AM and estrus females compared to diestrus females and males (Proano et al 2018), consistent with previous work between proestrus females and males (Wissman et al 2011). Relevant to this finding, sex differences in excitatory synapse number and associated markers have been identified between proestrus females and males (Forlano & Woolley 2010, Wissman et al 2012, Wissman et al 2011). The lack of an effect on excitatory

synapse properties in the present study is of especial interest given estradiol's rapid regulation of excitatory synapse mEPSC frequency and amplitude in female but not male AcbC (Krentzel et al 2019). Also relevant are anatomical experiments which have demonstrated estradiol-induced decreases in dendritic spine density in AcbC MSNs in female rats and hamsters (Peterson et al 2015, Staffend et al 2011). Several not necessarily mutually exclusive possibilities may explain why no differences in mEPSC properties were detected in response to the estradiol doses employed in this study. First, we note that no exogenous estradiol replacement paradigm in an ovariectomized female perfectly matches the natural *in vivo* conditions, however, great care was taken in the development of this paradigm to mimic the temporally cycling circulating estradiol concentrations across the estrous cycle (Scharfman et al 2007). Second, estradiol action on MSN excitatory synapse properties could be solely regulated by a rapid action of estradiol, which would be consistent with the results of Krentzel, Mermelstein, Becker and others (Hu 2006, Krentzel et al 2019, Song et al 2019). A rapid action of estradiol may not be detected by the current study, given that animals were sacrificed two hours after the last estradiol injection. This sacrifice time was chosen to more accurately mimic proestrus estradiol levels, and to match the previously validated protocol (Scharfman et al 2007). A third possibility is that there are temporal differences in the impact of estradiol on electrophysiological properties, especially between the rapid (seconds to minutes) and non-rapid (hours to days) timescales, and in response to repeated doses of estradiol, as has been demonstrated in other systems (Zakon 1998). This conjecture is consistent with previous work showing that AcbC mediated behaviors are sensitive to estrogen priming (Becker & Rudick 1999, Krentzel et al 2020). Fourth, other hormones also cycle, most prominently progesterone, and progesterone alone or in combination with estradiol may regulate glutamatergic synapse properties or other electrophysiological properties. Fifth, it

is always possible that a secondary factor may be present. One example could be stress and anxiety. Stress may sex-specifically influence glutamatergic synapse and estrogen receptor action in the nucleus accumbens core, as has been demonstrated in the nucleus accumbens shell (Brancato et al 2017, Hodes et al 2015, Lorsch et al 2018). While we have attempted to mitigate the impact of stress by performing extensive handling and saline-injection habituation before experimentation, formally this possibility cannot be ruled out.

Other potential factors of note include differences in estrogen sensitivity and/or sex differences between MSN subtypes (Cao et al 2018a), although the robust nature of MSN estrogen sensitivity and sex differences may argue against this particular limitation. Nevertheless, an important future experiment will be to determine which MSN subtypes are estrogen-sensitive, using techniques other than the transgenic mice that show minimal or no sexual differentiation in MSNs, at least during the prepubertal period (Cao et al 2018a, Willett et al 2019). These future experiments are all the more relevant given that MSN subtypes can differentially express dopamine receptors, including but not limited to the D1 and D2 dopamine receptors. AcbC MSN D1 and D2 dopamine receptors have been documented to underlie rewarding versus aversive behaviors, among many other functions (Calipari et al 2016). This is relevant to estrogen action as the expression of D1 and D2 dopamine receptors can be influenced by estradiol exposure (Chavez et al 2010, Le Saux et al 2006, Morissette et al 2008). Estradiol administration has been documented to reverse an ovariectomy-induced decrease in D2 receptor expression (Le Saux et al 2006). Female rats have been reported to exhibit greater expression of the D1-D2 heterodimer complex, a protein receptor complex known to induce depression-like and anxiety-like behaviors which could ultimately play a role in modulating MSN electrophysiological properties (Hasbi et al 2020, Shen et al 2015). Thus, there are likely

complex interactions between estradiol, dopamine, glutamate, and MSN electrophysiological properties.

While this discussion has focused upon ovarian-sourced estradiol action in the AcbC, *de novo* synthesis of this hormone by the enzyme aromatase may also be implicated in mediating electrophysiological properties in this brain region. There is some evidence that aromatase is active in the nucleus accumbens, although this area of research is relatively understudied (Krentzel & Meitzen 2018, Yoest et al 2018). Aromatase presence in the nucleus accumbens was detected in mice pretreated with Vorozole, an aromatase inhibitor known to increase the intensity of aromatase immunoreactivity in quail brains, as well in mice enhanced with green fluorescent protein (EGFP) that activated upon transcription of the *Cyp19A1* aromatase encoding gene (Foidart et al 1995, Stanić et al 2014). In addition, changes in nucleus accumbens gene expression accompany sex-specific suppression of spontaneous physical activity in aromatase knockout mice (Shay et al 2020). Functionally, in the caudate-putamen of male rats, at least one type of long-term potentiation in MSNs is mediated by aromatase-induced synthesis of estradiol, although females and the nucleus accumbens were not included in the study (Tozzi et al 2015). In female but not male nucleus accumbens core, estradiol rapidly regulates mEPSC frequency (Krentzel et al 2019). Building upon this evidence, we speculate that locally synthesized estradiol rapidly regulates the sexually differentiated MSN glutamatergic synapse properties, perhaps in concert with progesterone, while slower, ovarian-sourced estradiol action may modulate MSN membrane excitability. This speculation is similar to aspects of estrogen action on CA1 hippocampal dendritic spine density and synaptic plasticity function, which features influences of both ovarian- and locally sourced estradiol action (Grassi et al 2011, Kretz et al 2004, Prange-Kiel et al 2008, Woolley & McEwen 1993). Future studies can examine the

contribution of aromatase-induced estradiol synthesis in modulating AcbC excitatory synapse properties independently, or in concert with ovarian-sourced estradiol across the estrous cycle.

Given that we have established that estradiol induces profound differences in MSN electrophysiological properties, future research can also focus on elucidating the relevant receptor mechanisms. The adult AcbC exclusively expresses membrane-associated estrogen receptors (mERs) α, β, and GPER-1 (Almey et al 2015). While GPER-1 is a G-protein coupled receptor, membrane-associated ERα and ERβ are not. Instead, membrane-associated ERα and ERβ are palmitoylated and interact with caveolin proteins to couple with group I and group II metabotropic glutamate receptors (mGluRs) in the neuronal membrane to induce rapid hormone effects (Grove-Strawser et al 2010, Meitzen et al 2019, Meitzen et al 2013, Peterson et al 2016, Peterson et al 2015, Tonn Eisinger et al 2018b). In addition to rapid hormone effects, ERα and ERβ coupled with mGluRs can also activate second messenger signaling and long-term changes to neuron function (Meitzen & Mermelstein 2011). For example, the reported decrease in dendritic spine density in adult OVX rat AcbC upon estradiol administration is mediated by ERα and group I mGluR5 coupling as well as by mGluR-mediated endocannabinoid release and activation of CB1 receptors (Peterson et al 2016, Peterson et al 2015). In addition, AcbC-mediated behaviors and disorders such as anxiety and drug addiction have also shown estradiol sensitivity by mGluR5 activation (Martinez et al 2016, Martinez et al 2014, Miller et al 2020, Pomierny-Chamiolo et al 2017, Pomierny-Chamioło et al 2014). Thus, the differences in membrane excitability induced by estradiol could be mediated by membrane-associated ERα and ERβ (possibly via mGluR activation) and/or GPER-1. Finally, while our working model posits that estradiol acts on receptors in the AcbC itself to induce changes in MSN excitability, it is also possible that estradiol also acts on receptors in afferent brain regions, which then

transsynaptically signal to AcbC MSNs. To conclude, we believe that the findings of this manuscript provide a strong foundation upon which to pursue these future avenues of investigation.

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CHAPTER 5

Conclusion

This dissertation encompasses a comprehensive analysis of estrous cycle and ovarian hormone dependent modulation of nucleus accumbens core (AcbC) medium spiny neurons (MSNs). MSNs are the primary output neurons of the AcbC, a brain region critical for the cognitive processing of motivation, reward, and reinforcement. Here, I thoroughly tested and compared the differential expression of AcbC MSN excitatory synapse and intrinsic excitability properties across all phases of the rat estrous cycle and under the influence of estrous cycle relevant doses of estradiol. In addition, I investigated the association between circulating levels of estradiol and progesterone across the estrous cycle with AcbC MSN electrophysiological properties. The findings presented in this dissertation show that: 1) estrous cycle phase robustly modulates AcbC MSN excitatory synapse and intrinsic excitability properties; 2) removal of the estrous cycle via gonadectomy abolishes sex differences in AcbC MSN excitatory synapse and intrinsic excitability properties; 3) estradiol and progesterone differentially and synergistically influence the electrophysiology of AcbC MSNs; and 4) estrous cycle relevant doses of estradiol administered to ovariectomized female rats recapitulate the decrease in membrane excitability observed in naturally cycling female rats during the proestrus AM and estrus phases of the cycle. These data provide the first line of evidence for the underlying neuroendocrine mechanisms responsible for sex- and estrous/menstrual cycle-dependent differences in AcbC-mediated behaviors and disorders. Importantly, the research presented here demonstrates the necessity to include sex as a biological variable in basic neuroscience research and, when appropriate, assess estrous cycle stage in female animals.

Traditionally, basic neuroscience research has neglected to study the female brain (Beery & Zucker 2011, Mamlouk et al 2020, Will et al 2017). This has been a critical omission given that the hormonal milieu under which the brain operates profoundly differs between females and

males. In fact, out of all neuroscience articles published in six scientific journals in 2017, 44% did not use sex as a biological variable when females and males were included in the studies, 26% reported using male animals, and a dismal 5% reported using female animals only (Mamlouk et al 2020). This bias has had the unintended consequence of not only establishing the male brain as the 'standard' against which all brains are measured, but has also created a public health problem that limits the translational potential of basic neuroscience research for the medical care of women. Additionally, and in a practical level, the use of solely male animals and/or the disregard of sex as a biological variable hinders future discoveries on brain physiology. This limitation begs the question of why neuroscience has neglected to study females given its inherent potential for scientific discovery.

For decades, the predominant justification for excluding females from basic neuroscience research has been linked to the belief that females are more variable than males due to the estrous/menstrual cycle (Beery 2018, Galea et al 2020, Shansky 2019). The work presented by this dissertation shows that there is, indeed, variation across the estrous cycle in a brain region that is not involved in ovulation/reproduction *per se*. However, often ignored, is the fact that males produce the same hormones as females, albeit in different patterns and concentrations. Indeed, male humans and rodents actually experience diurnal fluctuations in testosterone levels that are controlled by circadian rhythm and social hierarchies (Albert et al 1986, Blanchard et al 1993, Ellis & Desjardins 1982, Giammanco et al 2005, Harden et al 2016, Kalra & Kalra 1977). Thus, the idea that hormonal fluctuations induce variability and are only relevant to females is inaccurate. Males also exhibit variation, but the underlying mechanisms may differ by sex, developmental time point, brain region, and measured endpoint. Indeed, the data collected for this dissertation qualitatively shows an overall equivalent variability spread between the data

collected from females and males. Even though there is variability across the estrous cycle in electrophysiological endpoints or between select phases of the cycle and males, in general, the estrous cycle does not necessarily create a robust sex difference in data variability *per se* (Chapter 2). Thus, these data strongly argue against the exclusion of female subjects because of the erroneous belief that females are more variable than males. Recent meta-analyses across various disciplines, including neuroscience, have exposed that the perception that there is greater variability in female data is incorrect. Surveys of physiological, cellular, hormonal, and behavioral measures have revealed that data collected from female mice, rats, and hamsters, three of the most used species in basic neuroscience research, are no more variable than data collected from males (Becker et al 2016, Prendergast et al 2014, Smarr et al 2017). In fact, no sex differences emerged in a study comparing variation between the sexes in several traits relevant for neuroscience such as behavior, histology, neurochemistry, and electrophysiology (Becker et al 2016). Thus, arguing against the exclusion of female subjects because of the erroneous belief that females are more variable than males.

Additionally, excluding females is both a lazy and a sexist excuse for ethical, medical, and scientific reasons. On the ethical front, the exclusion of females from basic neuroscience research is ethically and morally wrong because it further perpetuates archaic gender stereotypes that extend beyond the lab and move into society. Thus, supporting the status quo, i.e. excluding females, and directly perpetuating gender inequality. Regarding the medical front, implicit in scientific inquiry is the promise of advancing the human condition by developing new technologies and therapies to benefit health outcomes in both women and men. Neglecting to study females is contrary to this core goal because it ignores almost 50% of the population, who not coincidentally funds our research through their tax dollars. Therapies, drugs, and

technologies investigated in males do not always promote the health of women in the same way, if at all. Finally, excluding females from experiments based on the premise that they exhibit more variability simply inhibits scientific discovery. Had morphological data from female rats not been included in Catherine Woolley's seminal work on sex and estrous cycle regulation of hippocampal dendritic spine density (Woolley et al 1990, Woolley & McEwen 1992, Woolley & McEwen 1993), research into sex and estrous cycle effects in brain regions not directly associated with reproduction would have probably taken longer, or not been possible at all. Thus, the idea that females are too complicated to study is not only ethically and medically wrong but also scientifically unfounded.

Another reason often cited for the systematic exclusion of female subjects in basic neuroscience research is based on a claim that the inclusion of both sexes reduces statistical power. Neuroscientists have expressed concern that sample sizes might need to be doubled and/or quadrupled to identify treatment effects in studies using animals from both sexes and if the data from females is to be disaggregated by estrous cycle phase. Statistical analyses, however, have shown that factorial designs can identify main effects from treatment and sex with the same power as pairwise comparisons without having to necessarily increase sample size (Buch et al 2019, Collins et al 2014, Dayton et al 2016). In addition, the use of effect sizes rather than p-values can also aid in the understanding of sex effects (Beltz et al 2019, Ho et al 2019, Krentzel et al 2020). The only scenario in which loss in statistical power would result is if an interaction between treatment and sex is present. In that case, follow-up investigations should be designed to capture sex differences and their origins (Becker et al 2005). In her recent perspectives piece in Science magazine, Rebecca Shansky, argues that researchers should begin their studies with a mixed-sex pool of subjects and examine data by sex to detect sex differences.

If no sex differences are evident, then researchers can proceed analyses with mixed-sex cohorts. However, if sex differences are apparent, data should be disaggregated by sex and the origins of these differences should be studied (Shansky 2019).

The work presented in this dissertation resonates with the framework proposed by Dr. Shansky and others who have argued for the assessment of estrous cycle dependent differences under specific conditions. For decades, AcbC-mediated behaviors and disorders such as sexual motivation, addiction, and anxiety have exhibited sex- and estrous/menstrual cycle- dependent differences (Becker & Hu 2008, Becker et al 2001, Cummings et al 2014, Jenkins & Becker 2001, Krentzel & Meitzen 2018, Lebron-Milad & Milad 2012, Milad et al 2009, Miller et al 2020, Tonn Eisinger et al 2018, Yoest et al 2018). In addition, electrophysiological data from prepubertal and gonad-intact adult female rats shows a sex difference in mEPSC frequency favoring females (Cao et al 2016, Wissman et al 2011). Given these findings, it was necessary to elucidate the origins of these differences and if they were apparent across all stages of the estrous cycle. Here, I have not only shown that the sex differences reported in pre-pubertal rats persist into adulthood but are also maintained by the estrous cycle (Chapter 1). Indeed, AcbC MSN excitatory synapse properties and intrinsic excitability not only differ significantly across all stages of the estrous cycle but also disappear upon elimination of the estrous cycle via gonadectomy. These findings show a particular instance in which estrous cycle assessment served as a tool to investigate the origins of the sex differences reported in AcbC electrophysiological measures. Additionally, these findings served as the foundation to inspire the work presented in chapters 3 and 4 in which ovarian hormones, such as estradiol and progesterone, were probed to elucidate possible neuroendocrine mechanisms by which they modulate AcbC physiology. Through this work, I have shown that circulating levels of estradiol

and progesterone work independently and synergistically to influence AcbC MSN electrophysiology depending on the metric measured (Chapter 3). Excitatory synapse metrics, such as mEPSC frequency and amplitude, correlate independently with circulating levels of progesterone while intrinsic excitability metrics, such as resting membrane potential, the time constant of the membrane, and rheobase, correlate independently with circulating levels of estradiol. On the other hand, circulating levels of estradiol and progesterone together correlate with mEPSC amplitude, resting membrane potential, and input resistance in both the linear and rectified ranges. These data have the potential to 1) predict the direction of electrophysiological changes across the estrous cycle depending on circulating hormone levels, and 2) provide insight on the temporal action of estradiol and progesterone as their levels fluctuate across the estrous cycle. Finally, in chapter 4 I show that a time- and dose-dependent administration of a combination of estradiol benzoate and 17β-estradiol designed to mimic estradiol levels across the estrous cycle recapitulates the decreases in membrane excitability observed during the stages of the cycle that feature higher estradiol levels or effects, proestrus AM and estrus, respectively. These data show a causal role for estradiol modulation of AcbC MSN intrinsic excitability and provide the foundation for future investigations into the mechanisms through which estradiol regulates the ultimate output from this brain region. Importantly, in a broader context, my body of work serves as a roadmap for how to undertake estrous-cycle hypothesis driven questions. My work shows that staging the cycle and assessing hormone levels is not only feasible but simple.

Overall, my work shows that studying the female brain is important. Not only does this fulfill an ethical imperative but also a practical one. Studying the female brain unearths new avenues of research and offers the potential to inform translational and clinical work in certain human conditions such as drug addiction and mental health disorders, which exhibit disparities

between the sexes (Altemus et al 2014, Bale & Epperson 2015, Bao & Swaab 2010, Ferri et al 2018, Gobinath et al 2014, Goldstein et al 2019, Jurado-Coronel et al 2018, McCarthy 2016, Mendrek & Mancini-Marïe 2016, Valentino et al 2013). In addition, my dissertation provides a clear example of the importance of including sex as a biological variable in basic neuroscience research. By studying sex and the origins of sex differences, my dissertation not only enhances our knowledge of brain physiology but also shows that our work as neuroscientists should extend beyond the mere understanding of the brain. Concurrent with our desire to understand the brain, our work should also strive to advance health outcomes in both women and men, even if just to uphold the mission of our main funding agency, the National Institutes of Health, to "seek fundamental knowledge about the nature and behavior of living systems and the application of that knowledge to enhance health." More broadly, this dissertation calls into question the status quo in our field and proves that antiquated gender stereotypes should not permeate and inform our work on the bench. This is the only way that we will advance physical and mental health outcomes in both women and men.

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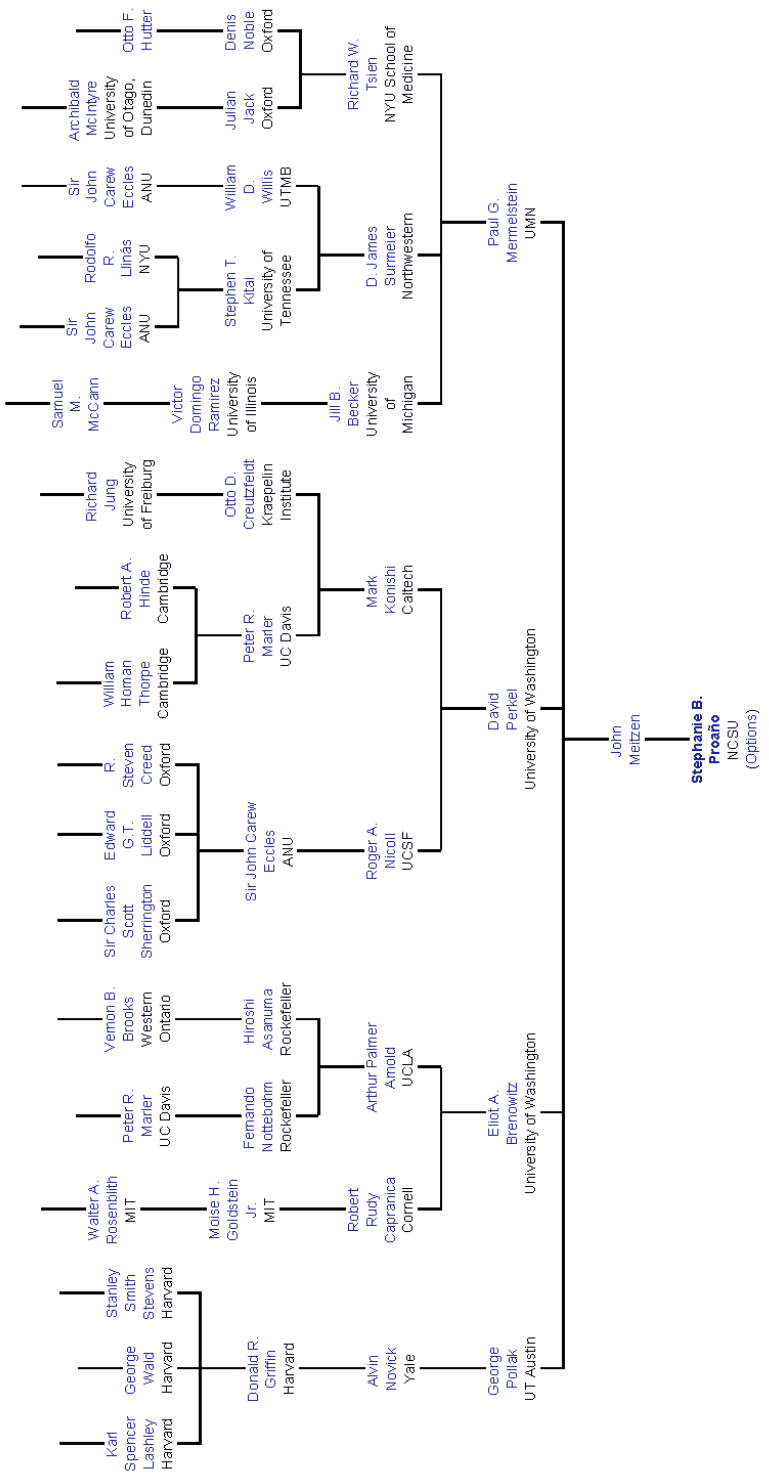
APPENDICES

Appendix A


Karl Spencer Lashley Harvard
George Wald Harvard
Stanley Smith Stevens Harvard
Donald R. Griffin Harvard
Alvin Novick Yale
Walter A. Rosenblith MIT
Moise H. Goldstein Jr. MIT
Robert Rudy Capranica Cornell
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Fernando Nottebohm Rockefeller
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