Stony Brook University

Assessment of gonadal hormone effects on the glutamatergic regulation of prefrontal cortical dopamine levels and executive cognitive function in rats

A Dissertation Presented

by

Mallory Locklear

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Neuroscience

Stony Brook University

August 2014

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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Schizophrenia, Parkinson's disease, depression, and anxiety, though very different disorders, all feature deficits in the executive functions of the prefrontal cortex. These deficits share properties of greatly reducing quality of life and being poorly responsive to available therapeutics. These cognitive problems also stem at least in part from an imbalance in dopamine control over the prefrontal cortex. Normally, the prefrontal cortex requires dopamine to be held at functionally optimal levels and when dopamine either rises above or falls below this range, prefrontal executive functions become impaired. The disorders in which executive cognitive domains are at risk thus comprise two groups – those that feature too much dopamine, or hyperdopaminergia and those where dopamine levels are deficient, i.e., hypodopaminergia. Strikingly, disorders resulting from prefrontal hypodopaminergia, such as schizophrenia, tend to affect men more than women, whereas women are more vulnerable to

hyperdopaminergic disorders, e.g., anxiety. This dissertation explores the neurobiological basis for sex differences in executive operations of the prefrontal cortex, and what makes men and women differentially vulnerable to executive dysfunction in disease. Using adult rats as animal models, my work directly compares male, female, and hormone-manipulated subjects on behavioral, neurochemical, and electrophysiological levels. This work reveals novel mechanisms through which gonadal hormones influence the ways that the male and female prefrontal cortices maintain functionally optimal prefrontal dopamine levels. More specifically, it shows that the relevant gonadal hormone impact is levied against intracortical, dopamine-regulating glutamatergic and GABAergic circuits in the prefrontal cortex itself, rather than its dopamine afferents. As current methods of treating cognitive symptoms in the executive domain are less than optimal, understanding how biological sex and sex hormones modulate these functions could yield an important advance. It is possible that the neurobiological differences that shape sex-specific vulnerability to prefrontal cognitive dysfunction in disease may lead the way to the development of more effective treatments for what are often disabling, treatment-resistant cognitive problems.

To Catherine, Jaime, and Michelle. My graduate school family.

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AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV:	D(-)-2-amino-5-phophonopentanoic acid
DA:	dopamine
E:	17β-estradiol
GABA:	gamma-Aminobutyric acid
GDX:	gonadectomy
GLU:	glutamate
NBQX:	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA:	N-methyl-D-aspartate
SN:	substantia nigra
OVX:	ovariectomy
PFC:	prefrontal cortex
TP:	testosterone propionate
VTA:	ventral tegmental area

General Introduction

The drive to understand the brain has led neurobiologists to study its every nuance, from neurons to human behavior. Interest in the more uniquely human behaviors associated with cognition and how these change in development, aging, and disease have led researchers to the prefrontal cortex (PFC), a brain region responsible for many of the highest order behaviors like decision making, planning, working memory, and impulsivity (Goldman-Rakic, 1987; Dalley et al., 2004). Studies in humans and animals alike have revealed that the function of the PFC and the complex, so-called executive functions it mediates are highly dependent on the neurotransmitter dopamine (DA) (Floresco and Magyar, 2006; Mehta and Riedel, 2006). One of the defining characteristics of the prefrontal cortex is its dense DA innervation (Berger et al., 1991) arising from nuclei in the ventral midbrain (Descarries et al., 1987). When the firing patterns of these DAergic cells are altered and DA levels in the PFC are disrupted, so too are the behaviors mediated by the PFC (Floresco and Magyar, 2006). Early studies in primates and rodents confirm the importance of DA to PFC by making the striking observations that selective lesions of these DA afferents produce behavioral deficits similar in magnitude to those produced by ablation of the PFC itself (Tassin et al., 1978; Simon et al., 1980; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Stam et al., 1989). Further, a number of neurological and neuropsychiatric diseases, such as schizophrenia, autism, Parkinson's disease, and others where deficits in PFC executive function are severe, appear to be coupled to abnormalities in PFC DA regulation (Sesack and Carr, 2002; Seeman, 2009; Neuhaus et al., 2010; Narayanan et al., 2013).

The work of my dissertation focuses on the PFC, its executive functions, and its DA dependence, and uniquely ties these to the additional characteristics of sex differences and sex hormones.

Sex differences in PFC executive functions have been broadly identified in humans and in animal models, and in the dysfunctions of the PFC associated with organic and preclinical models of disease (Mann et al., 1990; Leung and Chue, 2000; Jonasson, 2005; Miller and Cronin-Golomb, 2010; Lejbak et al., 2011). My work adds new dimensions to the extant literature describing research into how biological sex and gonadal hormones affect PFC DA homeostasis and DA-dependent PFC functions. Clearly, these questions are a synthesis of multiple concepts and constructs. The introductory sections below consider each of these separately, and provide further background detail for the PFC, its executive functions, its dependence on DA, and for the roles and mechanisms of sex and sex hormone impact on PFC physiology and function. In the final section, these pieces of background information are integrated into the bases for the specific research questions that are addressed in this dissertation.

The Prefrontal Cortex: A Synopsis of its Structure, Development, and Function

The prefrontal cortex lies anteriorly to the premotor and primary motor cortices and comprises the largest portion of the frontal lobe. In rats, the PFC consists primarily of so-called agranular regions i.e., cortical regions lacking a discernable layer IV (Ongur and Price, 2000). In this way, they differ from the PFC of monkeys and humans that include granular and dysgranular cortical areas (Ongur and Price, 2000). However, despite these cytoarchitectonic differences, inter-species homology has been

established on the bases of the connections between the PFC and other brain regions (Rose and Woolsey, 1948). For example, the reciprocal innervation between PFC and the mediodorsal thalamic nucleus is a feature that is common to the PFC of rats, monkeys, and humans (Leonard, 1969; Uylings and van Eden, 1990). Berger and others suggest that a dense DA innervation is among additional features that are common to the PFC of rodents, cats, and primates (Uylings and van Eden, 1990; Berger et al., 1991; Uylings et al., 2003).

In addition to inter-species parallels in PFC afferents and efferents, PFC development shares similarities across species as well. In both humans and rats, PFC development begins in utero and stands out as among the last brain region to fully mature (Berger et al., 1985; Berger et al., 1991; Gogtay et al., 2004). In rats, cell division in the PFC ventricular zone continues to take place postnatally (Van Eden and Uylings, 1985). Further, there is substantial dendritic and synaptic elaboration, and continued axon myelination that does not reach full maturity until early adulthood (Eayrs and Goodhead, 1959; Kalsbeek et al., 1988; Huttenlocher and Dabholkar, 1997; Andersen, 2003). The PFC's DA innervation also undergoes an unusual and unusually protracted developmental history. For example, superficial layers of the PFC receive DA innervation largely from substantia nigra projections and develop much later than deeper layers receiving input from the ventral tegmental area (VTA) (Berger et al., 1985; Descarries et al., 1987) and DA afferents continue to increase in density for up to two months after birth (Kalsbeek et al., 1988). Some speculate that this protracted development leaves the PFC vulnerable to pre and postnatal insults, such as exposure to teratogens, hypoxia, and birth trauma, and that this may be what renders its complex

functions especially vulnerable in developmental disorders such as schizophrenia, which has been linked to in utero and perinatal disturbances e.g influenza, birth-related hypoxia (Andersen, 2003).

Among the defining features of the PFC are the complex functions it mediates. The executive functions of the PFC have been defined as a coordinated effort of many smaller functions in order to achieve a larger goal (Funahashi, 2001; Elliott, 2003). These functions, including planning, working memory, behavioral flexibility, and impulsivity, rely on the health and maintenance of the PFC and clinical cases involving PFC lesions in humans have shown that damage to this part of the brain results in deficits of the executive functions (Glascher et al., 2012; Tsuchida and Fellows, 2013). Textbook examples include Phineas Gage who displayed drastic negative changes in personality after a large iron rod was driven through his PFC (Van Horn et al., 2012). Further, though the extents of functional PFC homology between primates and rodents are still debated, a number of tasks have been validated as tests of PFC constructs including working memory and flexibility, and deemed appropriate for investigating the neurobiological bases of these functions in rodent models (Castner et al., 2004; Chudasama, 2011; Fernando and Robbins, 2011). Using these tasks, experiments have shown that lesions in rodents model the PFC deficits observed in humans (Muir et al., 1996; Chudasama and Muir, 2001; Passetti et al., 2002; Chudasama and Robbins, 2004; Paine et al., 2013).

The impetus to understand executive functions and their neural bases comes not only from the drive to understand significant parts of human nature, but also because of the disabling consequences of PFC dysfunction when these occur in human diseases

and disorders. Some of the most poignant examples of disorders where deficits in PFC-mediated functions are prominent include schizophrenia, attention deficit hyperactivity disorder, major depressive disorder, and Alzheimer's disease (Ivanchak et al., 2012; Keefe and Harvey, 2012; Bora et al., 2013; Schrag et al., 2013; Snyder, 2013). In these, the links to PFC include evidence for reduced blood flow and glucose metabolism in the PFC, i.e. hypofrontality, changes in DA receptor expression, and differences in dopamine metabolism (Weinberger et al., 1986; Zametkin et al., 1990; Okubo et al., 1997; Egan et al., 2001). From personal to societal levels, these exact significant tolls. Studies have estimated that as much as 30 percent of the population suffers from some type of DSM IV-defined mental disorder (Kessler et al., 2005) and the World Health Organization found that in North America and Europe neuropsychiatric disease accounted for over 40 percent of the cumulative years lived with disability (Organization, 2003). The National Institutes of Mental Health estimated mental health costs in the United States including direct expenditures, disability benefits, and loss of earnings to exceed $300 billion in 2002 (Insel, 2008). Thus, the economic and human capital negatively impacted by disorders of the PFC is undeniable, as is the pressing need to better understand PFC mechanisms and mechanisms of PFC pathophysiology in disease. Further, that these disorders often include striking sex differences in onset, severity, symptomology, and treatment response highlights the necessity to determine how sex and sex hormones are involved in setting and maintaining PFC function. My dissertation focuses on elucidating the roles sex and sex hormones play in modulating PFC function and PFC-mediated behaviors. As described further below, what has

proven to be key to previous investigations of the PFC and its function as well as to the investigations reported in this dissertation is the DA innervation of the PFC.

Dopamine Innervation of the Prefrontal Cortex: Cells of Origin, Terminations, and Major Receptors

The DA innervation of the brain is anatomically widespread and originates from within a small number of subcortically located nuclei, which correspond to areas A8-A12 defined by Dhalstrom and Fuxe (1964). The DAergic innervation of the PFC that is the focus of my dissertation comes primarily from area A10, which corresponds to the VTA. Although the PFC also receives some additional DA input from the medial substantia nigra (A9), and retrorubral fields (A8), most of what is discussed in this dissertation relates to the VTA (Berger et al., 1985; Berger et al., 1991).

The arrangement of DA axons in the PFC differs somewhat in rats and primates (Berger et al., 1991). Although the prefrontal cortex is densely innervated by DA in both, in primates, PFC DA axons are found in nearly every cortical layer including a dense innervation to layer I and slightly less in layers II and III, whereas in rodents, the densest DA innervation targets layers V and VI (Berger et al., 1991). Further, whereas the laminar distribution of DAergic inputs to the PFC is largely set at birth in primates, these inputs are not fully matured in rodents until approximately 3 weeks postnatally (Berger et al., 1985). However, these anatomical and developmental differences are minor in comparison with the finding that in both rodents and primates the PFC relies heavily on its DA inputs – a feature that is central to this dissertation and is discussed further below (Berger et al., 1976; Berger et al., 1991; Ongur and Price, 2000).

Dopamine Dependence of the Prefrontal Cortical Functions

The DA dependence of PFC-mediated behaviors has repeatedly been established in both humans and animals. In humans, reduced PFC DA levels have been correlated with deficits in the executive functions mediated by the PFC in both healthy, aging adults as well as in diseases linked to DA dysregulation such as schizophrenia and attention deficit hyperactivity disorder (Davis et al., 1991; Goldberg et al., 2003; Arnsten, 2009; Seeman, 2009)Akil et al., 1999; Mattay et al., 2002; Backman et al., 2006; Cools and D'Esposito, 2011). Additionally, stress-induced elevations in PFC DA levels have been linked to deficits in PFC function including spatial working memory (Mizoguchi et al., 2000) and attentional set shifting (Nikiforuk, 2012). Animal studies have also shown that altering PFC DA tone, for example, through lesions of the DAergic inputs (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Stam et al., 1989) or application of DA receptor agonists or antagonists in the PFC (Zahrt et al., 1997; Winter et al., 2009), negatively impacts performances on tasks testing PFC function. Moreover, performances of rats with naturally low baseline accuracy levels on an attentional task were improved with DA receptor agonists while antagonists reduced performances of rats displaying high basal accuracy levels (Granon et al., 2000). Chronic stress has also been found to disrupt working memory due to DA dysregulation (Morrow et al., 2000; Moghaddam and Jackson, 2004) and dopamine receptor antagonists have been shown to prevent working memory deficits induced by stress-mimicking β-carbolines (Murphy et al., 1996). Thus, these studies

show that maintaining DA tone is key to PFC function and the mechanisms involved in preserving DA homeostasis are discussed below.

Mechanisms of Prefrontal Cortical Dopamine Transmission and Homeostasis

The ability to phasically change, restore, and tonically maintain intracortical DA levels is essential to PFC signaling and all need to be preserved in order for the PFC to function normally. Thus, mechanisms that contribute to DA dynamics and homeostasis are essential and have been the subject of much study. Some of the most potent mechanisms to have emerged from several investigated approaches are those that arise from within the PFC itself. More specifically, it has been shown that there are intracortical circuits that converge onto the PFC pyramidal cells in layer V and VI. These cells project to the VTA and control its activity (Karreman and Moghaddam, 1996; Carr and Sesack, 2000a). Because these VTA-projecting PFC cells project to and synapse onto DAergic (and non-DAergic) cells within the VTA (Carr and Sesack, 2000a), changing the activity of the PFC cells mono, di-, or polysynaptically modulates DAergic cell activity (Murase et al., 1993; Lodge, 2011). For example, stimulation of PFC-to-VTA projecting pyramidal cells has been shown to drive DAergic VTA cell firing (Murase et al., 1993; Lodge, 2011) and elevate intra striatal glutamate (GLU) and DA release (Karreman and Moghaddam, 1996; Harte and O'Connor, 2005). Moreover, there are multiple firing patterns of DAergic VTA cells – single spike and bursting – the latter classically defined by Grace and Bunney (1984). PFC stimulation not only increases single spiking firing rates, but can also induce cells to switch to bursting mode (Lodge, 2011). This is critical to the regulation of PFC DA tone and, therefore, to PFC

function for the following reasons. Under normal resting conditions, mesoprefrontal DA neurons of the VTA primarily exhibit single spike firing modes; this controls basal DA levels in the PFC (Grace and Onn, 1989; Overton and Clark, 1997). However, in response to PFC activation, salient stimuli, and others, these cells fire in bursts (Freeman and Bunney, 1987; Overton and Clark, 1997; Hyland et al., 2002). The switch to burst firing mode has been linked to substantial, albeit phasic, increases in PFC DA levels, which importantly remain elevated for upwards of minutes and undergo relatively widespread extracellular diffusion (Garris et al., 1993; Cass and Gerhardt, 1994). These are results, at least in part, of the paucity of dopamine transporters (DAT) in the PFC (Sesack et al., 1998). Although DA is also a substrate for the norepinephrine transporter (Yamamoto and Novotney, 1998), norepinephrine terminals are sparse in the deep layers of the PFC (Berger et al., 1976; Lindvall and Bjorklund, 1984).

With such prolonged actions and the need for precise levels, sensitive systems are needed in order to keep PFC DA under control. Microdialysis studies have shown that strong GLUergic mechanisms contribute to DA homeostasis. Thus, administration of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists into the PFC have resulted in decreases of PFC DA levels (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Aubele and Kritzer, 2012) while *N*-methyl-D-aspartate (NMDA) receptor antagonists produce increases in DA levels (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Aubele and Kritzer, 2012). The overall NMDA-mediated inhibitory effects are thought to be due to excitatory NMDA receptor actions acting through inhibitory interneurons in the PFC

(Homayoun and Moghaddam, 2007b). Further, there is also evidence that via different DA receptor families, DA itself can also influence PFC networks and contribute to its own regulation. Briefly, the actions of DA in the PFC, as in all brain regions, are complex, dictated in part by receptor subtype activations. The D1-like receptor family includes D1 and D5, while the D2-like receptor family includes D2, D3, and D4. The D1-like receptors are coupled to Gs proteins, which ultimately lead to an increase of the second messenger cyclic adenosine monophosphate (cAMP) while D2-like receptors couple to Gi proteins, which inhibit cAMP (Stoof and Kebabian, 1981). Further, while D1 receptors are more often localized on nonpyramidal cells and D2 is localized to pyramidal cells and interneurons (Vincent et al., 1993; Gaspar et al., 1995; Vincent et al., 1995), a small portion of nonpyramidal cells contain both D1 and D2 receptors (Vincent et al., 1993). O'Donnell and others have shown that D1 regulates PFC DA in an excitatory manner while D2 exhibits an overall inhibitory effect thought to be mediated by GABAergic interneurons (Wang and O'Donnell, 2001; Tseng, 2004).

Thus, overall DA tone and the phasic changes arising from behaviorally relevant stimuli play a modulatory and rather complex role in the PFC. This dissertation couples PFC DA homeostasis to sex and sex hormones and the related sex differences in PFC function, which are discussed in the following section.

Sex Differences in Prefrontal Cortical Functions

Prefrontal cortical functions are characterized by significant sex differences that occur in human development, healthy adult life, and in disease. For example, in humans, females display superior verbal fluency from childhood through adulthood

(Mann et al., 1990; Weiss et al., 2003; Herlitz et al., 2013) while males consistently outperform females on spatial tasks (Lejbak et al., 2011; Talarowska et al., 2013). In rats, females have been found to be less impulsive (Jentsch and Taylor, 2003; Spivey et al., 2009; Bayless et al., 2012; Bayless et al., 2013) while males have been found to have superior attentional processes (Jentsch and Taylor, 2003; Bayless et al., 2012).

Interestingly, deficits in PFC-dependent functions seen in diseases such as Parkinson's disease and schizophrenia also display sex differences in incidence, age of onset, symptomology, and response to treatment (Leung and Chue, 2000; Miller and Cronin-Golomb, 2010; Vaskinn et al., 2011; Han et al., 2012). The dimorphisms in disease and the potential benefits of understanding the underlying mechanisms highlight some of the most compelling reasons to pursue these areas of investigation. However, most of what is known about PFC function and DA homeostasis comes from experiments performed on male rats only. That strong sex differences exist in both humans and rodents in healthy PFC function as well as its dysfunction in disease highlights the necessity to more fully understand PFC function in *both* sexes. This dissertation reports new findings of sex differences in both PFC DA modulation and PFC-dependent behaviors along with previously unobserved roles for both androgen and estrogen. These gonadal hormones, which likely contribute to both the previously observed and the newly reported sex differences of this dissertation, are discussed in relation to PFC function below.

Gonadal Hormones and Prefrontal Cortical Functions

In humans, performances on a number of PFC-dependent spatial tasks such as mental object rotation and block design have been shown to be positively correlated with circulating androgens in healthy adult and aged men (Gordon and Lee, 1986b; Christiansen and Knussmann, 1987; Janowsky et al., 1994; Hooven et al., 2004) as well as with the hormone flux that occurs across the menstrual cycle in women (Hampson, 1990a; Hausmann et al., 2000; Halpern and Tan, 2001). Similarly, poor performance on PFC-dependent tasks have also been found to correlate with low levels of testosterone in men with Klinefelter's syndrome (Patwardhan et al., 2000) or in patients with prostate cancer undergoing androgen deprivation therapies (Nelson et al., 2007). Further, the severity of cognitive/PFC deficits in Parkinson's and, Alzheimer's disease, and the negative symptoms of male schizophrenic patients have all been found to be correlated with low levels of testosterone in diagnosed males (Okun et al., 2002; Okun et al., 2004; Cherrier et al., 2005). Similarly, estrogen replacement/augmentation therapy has been shown to improve cognitive deficits in women diagnosed with Alzheimer's disease (Henderson et al., 1994; Paganini-Hill and Henderson, 1994) while the severity of positive symptoms in diagnosed female schizophrenics have been found to be inversely related to fluctuating estrogen levels across in the menstrual cycle (Bergemann et al., 2007; Seeman, 2012).

Corresponding effects of hormones have been observed in animals. For example, in female rats, performances on working memory tasks such as the radial arm maze and Morris water maze have been found to fluctuate across the estrous cycle (Warren and Juraska, 1997; Healy et al., 1999; Pompili et al., 2010). However, while intriguing, it must be borne in mind that correlations with hormone levels do not speak to

causation. Rather, it is only with experimentally controlled hormone deprivation and replacement that one can ascribe causal roles to gonadal steroids in cognitive processes of the PFC. In ovariectomized (OVX) female rats and gonadectomized (GDX) male rats, both deprivation paradigms have been shown to impair performance in operant (e.g., alternating lever press, delayed match, and non-match to sample) and spatial maze testing (e.g. radial arm maze, T-maze, Y-maze, Morris water maze, etc.) of PFC function (Bimonte and Denenberg, 1999; Kritzer et al., 2001; Sato et al., 2003; Kritzer et al., 2007; Gibbs and Johnson, 2008; Spritzer et al., 2008). Further, while OVX-induced deficits have been shown to be attenuated by estradiol supplementation (Luine et al., 1998; Bimonte and Denenberg, 1999; Gibbs and Johnson, 2008), GDX-induced impairments have generally been found to be androgen-, but not estrogen-sensitive (Kritzer et al., 2001; Sandstrom et al., 2006; Kritzer et al., 2007; Spritzer et al., 2011). My dissertation builds on these behavioral data and adds findings of gonadal hormone effects on the PFC DA systems on which these functions are reliant. As discussed below, the background for this comes primarily from studies in male rats alone.

Androgens and Prefrontal Cortical Dopamine Systems

In addition to the behavioral deficits observed following GDX, potentially related/relevant anatomical and physiological changes in PFC DA systems have been observed as well. More specifically, in long-term GDX rats, immunohistological studies have shown increased densities of axons immunopositive for the DA synthesizing enzyme tyrosine hydroxylase (Kritzer, 2003). Additionally, microdialysis studies have

shown that GDX increases basal extracellular DA levels in the medial PFC by nearly two-fold (Aubele and Kritzer, 2011). These GDX-induced changes were all found to be androgen sensitive and estrogen insensitive. This converges with behavioral effects in the adult male GDX rat model and led to intensive efforts to uncover mechanisms of androgen regulation of PFC DA levels. After ruling out effects on DA uptake (Meyers and Kritzer, 2009), DA catabolism (Meyers et al., 2010), or a likely androgen regulation of mesoprefrontal DA neurons themselves, an unsuspected candidate was found in the intracortical GLU mechanisms described above that regulate PFC DA levels (Aubele and Kritzer, 2012). These findings, described below, are foundations for the work presented in this dissertation.

Androgen Regulation of Glutamatergic Influences on Prefrontal Cortical Dopamine

Recent work in the Kritzer lab has applied classical microdialysis drug delivery assessments of the glutamatergic influences on PFC DA levels pioneered by Feenstra, Moghaddam, and others (above) to the GDX rat model. Recall that while foundation studies in males showed that AMPA has an overall excitatory influence on PFC DA levels and NMDA an inhibitory influence, fundamental differences were found in GDX rats. For example, following reverse dialysis delivery of the AMPA antagonist NBQX into the medial PFC, extracellular PFC DA levels in GDX rats failed to show the expected decreases (Aubele and Kritzer, 2012). Further, following infusion of the NMDA antagonist APV, DA levels in GDX rats paradoxically decreased rather than increased as expected (Aubele and Kritzer, 2012). These effects were androgen-, but not estrogen-sensitive. Having also found that the VTA-projecting pyramidal cells of the

PFC were significantly androgen receptor-enriched (Aubele and Kritzer, 2012), a working model that I have used to formulate and interpret studies of this dissertation was proposed (Fig In. 1). Normally, in male rats, the primary locus of NMDA-mediated regulation of PFC DA levels occurs through inhibitory interneurons (Homayoun and Moghaddam, 2007b). The aberrant outcome in GDX rats is explained by a model wherein circulating androgens present in the intact male tonically suppress the NMDA-sensitivity of VTA-projecting PFC pyramidal cells. However, when circulating androgen levels are low, as they are in GDX rats, the model suggests that VTA-projecting pyramids will become susceptive to activation by NMDA agonists. In essence, a decrease in androgen level shifts the DA-influence of NMDA receptor stimulation on PFC DA levels from an inhibitory to an excitatory action (Fig In. 1). It was reasoned that this shift increases the descending drive from the PFC onto mesoprefrontal DAergic cells and explains the tonically elevated basal PFC DA levels observed in GDX rats.

If true, such androgen control over the NMDA-mediated influence on PFC DA levels could have significant implications for understanding and controlling disease and an NMDA receptor hypofunction model of schizophrenia, currently a prominent hypothesis describing the neurobiological underpinnings of this disorder (Laruelle, 2014), is premised on intracortical NMDA receptor-mediated effects being as per intact males. As will become evident in this dissertation, understanding the roles that sex and sex hormones play at this pivotal site may require a substantial change in current thinking.

Questions Addressed in this Dissertation

Despite repeated observations of sex differences in disease states, men and women are often treated with the same therapeutic drugs. For cognitive symptoms this has proven to be a failure and no treatments are particularly effective in either sex. It seems that sex differences are giving a clue to the biology that could the pave way to improved therapeutic approaches. Still, foundational work in animal models is surprisingly limited. Despite clear observations of sex differences resembling those found in humans, they have not been thoroughly addressed. Further, there are two main areas with significant gaps. On the one hand, what is known about the basic neurobiology and disease models comes from studies mainly using male subjects; in many cases, it has never even been considered that the female brain could hold significant/fundamental differences. Second, when it comes to hormones, females dominate experimental studies, with very little known about the hormone effects in males. My dissertation focuses on PFC-mediated cognition with specific goals to contribute to both of these gaps. Thus, the majority of studies described use male, female, GDX, and GDX rats supplemented with either testosterone propionate (TP) or 17β-estradiol (E).

In approaching the broad questions on the neurobiology of cognition, this dissertation hones in on a working model describing potential androgen effects on PFC DA regulation (Fig In. 1). Thus, in intact males rats, circulating androgens suppress the NMDA receptor-mediated excitability of VTA-projecting pyramidal cells. However, in GDX rats with significantly depressed androgen levels, the NMDA sensitivity of the PFC pyramidal cells is elevated, causing a shift of the overall NMDA-mediated influence on PFC DA levels from an inhibitory one to an excitatory one – an effect rescued by TP but

not E (Fig In. 1). Essentially, this model predicts both sex and hormone effects on NMDA actions and the cellular sphere of its intra-PFC influences on PFC DA levels. Under conditions of high androgen (intact male, GDX-TP rats), influence will be on the GABAergic interneurons and under conditions of low androgen (GDX, GDX-E, and female rats) the influence will localize to pyramidal cells (Fig In. 1). The individual chapters of the dissertation test certain predictions and assumptions that relate to this model.

Chapter I describes microdialysis studies that were conducted in order to test the model's tenet that NMDA actions experience fundamental shifts in the primary locus of NMDA influence from the inhibitory interneuron to the VTA-projecting pyramidal cell. In order to test this, NMDA drug challenge was combined with pharmacologic blockade of the major tonic influences of GABAergic interneurons. If the model is correct and the locus of NMDA influence on PFC DA regulation has indeed shifted to the VTA-projecting pyramidal cells in cases of low circulating androgens, i.e. in female, GDX, and GDX-E rats, then the NMDA antagonist should still affect PFC DA levels in these animals. Conversely, in rats with comparatively high circulating androgen levels, i.e. male and GDX-TP rats, obstruction of the inhibitory influence of the GABAergic interneurons should prevent an effect of NMDA receptor antagonists.

Chapter II describes tests of the electrophysiological implications of the working model for sex and sex hormone influences on NMDA receptor-mediated PFC DA regulation in the two major structures and cells involved: the VTA-projecting pyramidal cells of the PFC and the DAergic cells of the VTA. As a major excitatory influencer, NMDA alterations in brains where androgens are high or low are predicted to have

significant, cell-specific consequences for basal activity in both loci, and to have different sensitivity to pharmacological NMDA challenge. Accordingly, assessments of spontaneous activity, mean firing rates, and bursting activity were compared across groups. The working model predicts elevated activity in GDX and GDX-E rats and potentially explains previous findings of GDX-induced, androgen sensitive, estrogen insensitive elevations in PFC DA levels. Further, NMDA receptor-mediated effects were tested in studies where recordings of DAergic VTA cells were performed in the presence of intra-PFC infusion of an NMDA antagonist.

The final chapter (Chapter III) describes studies that establish the working model and the sex and sex hormone impacts on PFC DA regulation as relevant to cognitive behavior. The Barnes maze is used first to establish it as a new paradigm for assessing sex and sex hormone effects on behavioral constructs of spatial working and spatial reference memory and additionally as a platform for demonstrating NMDA as the pivot point for behaviorally relevant hormone influences on PFC physiology (Chapter II) and DA levels (Chapter I). The working model predicts that the elevated NMDA receptor-mediated excitability of VTA-projecting pyramidal cells and the resulting disruptions in PFC DA regulation in GDX and GDX-E rats will lead to deficits in Barnes maze performance that may be rescued through intra-PFC infusion of an NMDA antagonist.

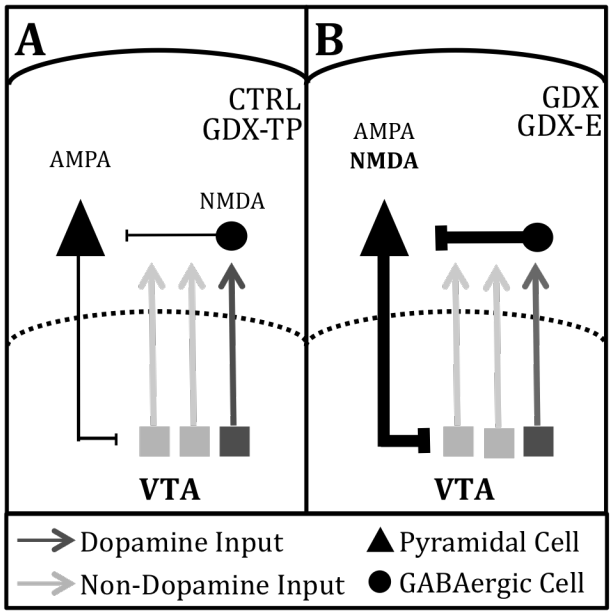

Figure In. 1. Circuit diagram of a working model for differences in glutamatergic influences on PFC DA levels in control (CTRL) and GDX-TP (A) and GDX and GDX-E (B) rats. (A) In CTRL and GDX-TP rats, approximately one-third of the VTA-to-PFC projecting cells (gray squares) are DAergic (dark gray square). The activity of these cells are regulated by AMPA-mediated excitatory actions on descending pyramidal cells (black triangle) that project down to the VTA and by NMDA-mediated suppressive effects acting through GABAergic interneurons (black circle) within the PFC. (B) In GDX and GDX-E rats, AMPA-mediated excitation of descending pyramidal cells is reduced compared to males. Additionally, NMDA-mediated effects act primarily through excitatory actions on pyramidal cell activity and increase the overall excitability of these projections.

Chapter I

Sex differences and gonadal hormone effects distinguish intracortical glutamate and GABA receptor-mediated regulation of extracellular dopamine levels in the prefrontal cortex of adult rats

As described in the General Introduction, the prefrontal cortices (PFC) of humans and animals mediate higher-order executive functions including working memory, behavioral flexibility and decision-making (Goldman-Rakic et al., 1990; Dalley et al., 2004; Tandon, 2013). Prefrontal dysfunction also underlies the cognitive deficits seen in a number of neurological disorders including Parkinson's disease and schizophrenia (Dubois and Pillon, 1997; Zgaljardic et al., 2003; Eisenberg and Berman, 2010; Ortiz-Gil et al., 2011; Narayanan et al., 2013). These executive operations rely on intracortical PFC dopamine (DA) levels being maintained within functionally optimal ranges, (Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Landau et al., 2009; Cools and D'Esposito, 2011) and disease processes, drug stimulation and other factors that move PFC DA levels outside of these limits produce deficits in executive information processing (Davis et al., 1991; Goldberg et al., 2003; Javitt, 2007; Arnsten, 2009; Scott and Aperia, 2009; Seeman, 2009). The directions from which the work of this dissertation approaches questions about the neurobiology of PFC are from perspectives of the sex differences that are well known to characterize both executive functions and the incidence and/or severity of executive dysfunctions in disease (Goldman et al., 1974; Roof et al., 1993; Overman et al., 1996; Lacreuse et al., 1999;

Leung and Chue, 2000; Faraji et al., 2010; Miller and Cronin-Golomb, 2010; Woolley et al., 2010; Mendrek and Stip, 2011). At their heart, my experiments ask whether sex differences in PFC operations might be related to sex differences in the functionally critical DA systems that underlie them.

Recent tract tracing studies in rats have shown that among the mesoprefrontal projections from the VTA there is a nearly two-fold female over male difference in the proportions of afferents that are DAergic (Kritzer and Creutz, 2008). However, basal PFC DA levels measured in tissue homogenates have been found to be either similar across sex (Tanila et al., 1994; Duchesne et al., 2009) or higher in males (Dalla et al., 2008). This suggests the possibility for sex differences in the regulatory mechanisms that set functional PFC DA levels. As described in the General Introduction, these mechanisms include strategically placed, intracortical receptor subtype-specific glutamatergic (GLU) influences that tonically and flexibly regulate DA levels (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Del Arco and Mora, 1999; Wu et al., 2002; Aubele and Kritzer, 2012). Recent studies using *in vivo* microdialysis and reverse dialyses drug challenge have shown that these PFC DA-regulating GLU mechanisms are sensitive to changes in circulating gonadal hormone levels in adult male rats (Aubele and Kritzer, 2012). Specifically, GDX reduces the excitatory effects of AMPA on PFC DA levels and switches the NMDA-mediated influence on DA levels from inhibitory to excitatory, effects attenuated by androgen and not estrogen (Aubele and Kritzer, 2012). These are the foundation data for the development of the working model and the shift in NMDA site of action. This chapter extends the prior data and tests the model in three fundamental ways. First, I asked whether GABA receptor

subtype-specific DA homeostatic mechanisms are hormone sensitive in males; secondly, I extended the GLU And GABA studies to females to find if they differ across sex, and finally, I used GABA blockade to determine if NMDA-regulation is differentially localized at or upstream of PFC interneurons in males, females, GDX, and hormone-replaced groups as the model predicts.

Previous studies—all performed exclusively in the male brain, have shown that tonic regulation of PFC DA tone is achieved in part by a balance of offsetting receptor-subtype-specific intracortical GLU influences (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Del Arco and Mora, 1999; Wu et al., 2002; Balla et al., 2009; Aubele and Kritzer, 2012) with phasic but surprisingly little tonic influence provided by GABAergic signaling (Yonezawa et al., 1998; Balla et al., 2009). Thus, through a complex targeting of PFC interneurons and/or pyramidal cells, intracortical GLU systems have been shown to work in concert to control the PFC's descending influence over the firing of mesoprefrontal DA neurons and maintain PFC DA homeostasis. More specifically, studies combining techniques of *in vivo* microdialysis or electrophysiology with drug challenge have shown that *N*-Methyl-D-aspartate (NMDA)-mediated GLU actions engage PFC interneurons to inhibit the PFC's descending drive over the ventral midbrain and tonically suppress PFC DA levels (Aubele and Kritzer, 2012; Del Arco and Mora, 2002; Homayoun and Moghaddam, 2007b; Jackson et al., 2004; Povysheva and Johnson, 2012). In contrast, local α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-mediated systems excite PFC pyramidal neurons, including those projecting to the ventral tegmental area and tonically stimulate mesoprefrontal DA neurons and DA release back in the PFC (Jedema and Moghddam, 1996; Takahata

and Moghaddam, 1998; Wu et al., 2002; Aubele and Kritzer, 2012). Together, with what have largely been shown to be phasic, resetting actions of intracortical GABA-A and GABA-B receptor mediated influences (Yonezawa et al., 1998; Balla et al., 2009; Del Arco et al., 2011), these regulatory processes play important roles in modulating electrophysiological properties (Tong et al., 1996; Wang et al., 2010; Povysheva and Johnson, 2012) and complex behaviors (Aultman and Moghaddam, 2001; Feenstra et al., 2002; Fejgin et al., 2009) associated with the PFC, and have been repeatedly implicated in the hyper- and hypodopaminergia associated with PFC dysfunction in disease (Lewis et al., 1999; Cryan and Kaupmann, 2005; Kehrer et al., 2008; Kantrowitz and Javitt, 2010; Gonzalez-Burgos and Lewis, 2012; Lewis et al., 2012). Hypothesizing that sex differences in the organization and/or operations of these circuits enables the structurally different mesoprefrontal DA systems of the male and female brain to maintain similar, functionally optimal PFC DA levels, one part of this chapter describes results from studies combining *in vivo* microdialysis with reverse dialysis administration of receptor subtype-selective GABA (GABA-A, GABA-B) and GLU (NMDA, AMPA) antagonists and HPLC with electrochemical detection to quantitatively compare drug effects on DA levels in the PFC of adult male and female rats (estrous cycles tracked). In keeping with overall objectives of describing hormone effects in the male brain, the second part of this chapter tests the GABAergic influence on PFC DA levels in GDX males, not previously explored. Finally, to further test the model and to determine if localization of NMDA influence is sensitive to sex and sex hormones, studies combined *in vivo* microdialysis with a novel dual-drug challenge paradigm to isolate GABA from

the circuit and determine whether it was necessary for NMDA receptor-mediated effects on PFC DA homeostasis in these groups.

Materials and Methods

Animal Subjects

A total of 89 adult male and 53 female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Animals were housed in a specific pathogen-free environment in same sex pairs under a 12/12 h light/dark cycle (lights on at 0700) with food (Purina PMI Lab Diet: Prolab RMH 3000) and water available *ad libitum*. Animals weighed between 200 and 400 grams at the times of the microdialysis studies. For these studies, 7 female (5 in diestrus, 2 in proestrus determined by vaginal lavage), 7 male, and 4 GDX rats were infused with 5-50 μM of the competitive GABA-A antagonist bicuculline; 17 female (15 in diestrus, 1 in proestrus, and 1 in estrus), 20 male, 5 GDX, 6 GDX-E, and 4 GDX-TP rats were infused with 30-60 μM of the GABA-B antagonist CGP52432; 8 females (6 in diestrus, 2 in proestrus) and 8 males were infused with 50-150 μM of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo [f] quinoxaline-2,3-dione (NBQX); 16 females (13 in diestrus, 2 in proestrus, and 1 in estrus) and 13 males were infused with 100-670 μM of the NMDA antagonist D(-)-2-amino-5-phosphonopentanoic acid (APV); 5 females (3 in diestrus, 1 in proestrus, and 1 in estrus), 5 males, 5 GDX, 7 GDX-E, and 5 GDX-TP rats were co-infused with 50 μM CGP52432 and 500 μM APV. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize animal use and discomfort.

Surgeries

Twenty-eight days prior to microdialysis, male rats underwent GDX or sham surgery. Both surgical procedures were performed under aseptic conditions using intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. For sham and GDX surgeries, an incision was made into the scrotum. For GDX, the vas deferens was bilaterally ligated and both testes were removed. For hormone-supplemented animals slow-release pellets containing either testosterone propionate or 17β-estradiol (Innovative Research of America, Sarasota, FL) were implanted within the tunica. All incisions were closed with wound clips, which were removed after 10 days. Rats were given subcutaneous injections of buprenorphine (0.03 mg/kg) post operatively before being returned to home cages.

Hormone Replacement

Male rats were implanted with slow release pellets at the time of GDX. The testosterone propionate (TP) pellets used released 3-4 ng of TP per milliliter of blood per day and the 17β-estradiol (E) pellets used released 25 pg of E per milliliter of blood per day; both have been used previously in this and other labs and have been shown to produce sustained plasma hormone levels falling within physiological ranges (Collins et al., 1992; Adler et al., 1999; Kritzer, 2000). The efficacies of GDX and hormone replacement were verified in quantitative analyses of the weights of animals' androgen-sensitive bulbospongiosus muscles (BSMs) (Wainman and Shipounoff, 1941).

Stereotaxic Placement of Guide Cannulae

Craniotomies were performed 24 hours before the microdialysis experiments under aseptic conditions and using intraperitoneal injections of ketamine (0.9mg/kg) and xylazine (0.5 mg/kg) as anesthesia. Stereotaxic coordinates were used to place guide cannulae (14mm, CMA Microdialysis, North Chelmsford, MA) within the left pregenual medial PFC. Cannulae were secured to the skull with shallow anchor screws and dental cement. After surgeries, animals were given single doses of buprenorphine (0.03 mg/kg) and returned to home cages for recovery.

In vivo Microdialysis and HPLC/EC Analysis

Microdialysis studies took place during animals' subjective nights. Animals were typically asleep for the duration of the experiment. Rats were placed in clear bowls (Raturn, BioAnalytical Systems) and allowed to acclimate for 10 min. Microdialysis probes (100,000 Da cutoff, 2mm PES exposed membrane tip, CMA Microdialysis) were then gently inserted through guide cannulae and perfused with artificial cerebrospinal fluid (aCSF) (145mM NaCl; 2.8 mM KCl; 1.2 mM $MgCl_2$; 0.25 mM ascorbic acid; 5.4 mM D-Glucose, 1.2 mM $CaCl_2$ in 1L H_20, pH 6.8) at a flow rate of 2 µL/min for a 2 hour equilibration period. Next, baseline dialysates were collected (10 µl) and directly injected into the HPLC every 15 min using an online autoinjector (Pollen-8, BAS). After obtaining 3 consecutive stable baseline measures (DA levels within 5% of each other), drug was added to the aCSF [5-50 µM bicuculline (Sigma-Aldrich Chemical Co.), 30-60 µM CGP52432 (Tocris Bioscience), 100-150 µM NBQX (Tocris Bioscience), 100-670 µM APV (Sigma-Aldrich Chemical Co.), or 50-200µM picrotoxin (Sigma-Aldrich

Chemical Co.)] and infused for 120 min while dialysates were collected (every 15 min); exceptions included the co-administration study where CGP52432 was administered for 45 min followed by a 90 min co-infusion of CGP52432 and APV and the dose response studies in which each drug concentration of APV was administered for 75 min and each concentration of CGP52432 was administered for 45 min. With the exception of the dose response studies, after drug delivery, infusion of aCSF was resumed and dialysates were collected until DA levels returned to within 5% of pre-drug baseline values. It should be noted that some of the animals in each of the dose response studies did not receive each drug concentration as problems sometimes arose due to the very long experimental timelines of these studies.

Estrous Stage Determination, Euthanasia, Histology and Determination of Probe Placement

At the conclusion of microdialysis studies, female rats were vaginally lavaged and vaginal cytology was used to determine estrus cycle stage (Marcondes et al., 2002; Goldman et al., 2007). All rats were euthanized by rapid decapitation. Brains were removed and post-fixed for 2-4 days in a 10% buffered formaldehyde solution containing 30% sucrose for cryoprotection. Once fixed, brains were rapidly frozen in powdered dry ice and serially sectioned in a coronal plane on a freezing microtome (40 µm). A 1 of 4 series sections taken from the level of mid-olfactory bulb to the genu of the corpus callosum were slide mounted and counter-stained with 0.5% cresyl violet (Fig 1.1). Light microscopic evaluation was used to map probe tracks in relation to cortical cytoarchitecture. Only those cases where dialysis probes were confirmed to

have spanned the deep layers of the left prelimbic and infralimbic medial PFC were included in the analysis (Fig 1.1).

Data Analysis

Microdialysis: DA concentration

Dialysate samples (10 μL) were directly injected into an HPLC system (PM 92-E pump, BAS, West Lafayette, IN) and analyzed using a microbore column (UniJet, 1.0 mm inner diameter, 100 mm length, 3 μm Octadecylsilane particles; BAS) and a BioAnalytical Systems LC-Epsilon detector (BAS). The E_{app} was +0.65 V versus the Ag/AgCl reference electrode. The mobile phase consisted of 14.5 mM NaH_2PO_4; 30 mM sodium citrate; 10 mM diethylamine HCl; 2.2 mM 1-octanesulfonic acid; 0.027 mM ethylenediaminetetraacetic acid; 7.2% acetonitrile (v/v); 1% tetrahydrofuran (v/v), pH 6.0 (with phosphoric acid). All chemicals used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

DA peaks were isolated and quantified (ng/mL) in relation to a series of DA standards of known concentrations (2, 10 ng/mL) run before and after each microdialysis study. Dopamine concentration (fmol/μL) was calculated using ChromGraph Software (BAS) and measurements of “peak area”. Drug-induced changes in DA level were also quantified as a percent of pre-drug baseline. Probe efficiency was determined to be 10-18%, and an overall detection limit of 8 fmol was achieved.

Statistics

Basal DA levels were compared across groups using a one-way analysis of variance (ANOVA). Drug effects on PFC DA levels were evaluated using two-way ANOVAs with repeated measures design where sex served as the independent factor and the 15 min sample bins as the repeated measure. When significant sex, time, or sex by time interactions were found, post hoc Bonferroni analyses were used to identify when along study timelines drug effects on PFC DA levels significantly diverged between males and females. Additionally, within-sex one-way ANOVAs with repeated measures design, with the 15 min sample bins serving as the within-subjects factors, were run to determine at which time point during drug administration DA concentrations were significantly different from baseline. In all cases $p < 0.05$ was accepted as significant and $0.05 < p \leq 0.09$ was designated as near significant.

Results

Basal Extracellular PFC DA Levels in Male Rats and Female Rats Across the Estrous Cycle

Basal extracellular PFC DA levels measured prior to drug delivery in males rats ranged from 0.039-0.504 fmol/μL and had a group average of 0.147 fmol/μL (± 0.021 SEM, Fig 1.2). A similar range of DA levels were found in female rats in diestrus (from 0.031-0.412 fmol/μL) but their group average was slightly lower (0.121 fmol/μL ± 0.020 SEM) than males. Additionally, DA levels in the female rats in proestrus ranged from 0.024-0.238 fmol/μL and had a group average of 0.089 fmol/μL ± 0.021 SEM (Fig 1.2). Finally, DA levels in the female rats in estrus ranged from 0.019-0.118 fmol/μL and had the lowest group average (0.064 fmol/μL, ± 0.029 SEM, Fig 1.2). Statistical

comparisons of the data (one-way ANOVA) were carried out between males and all females as well as between males and females separated by estrous cycle stage. None of these comparisons identified significant main effects of group. Additionally, although the numbers of subjects in the proestrus and estrus groups were low, there were no clear indications of consistent differences between DA levels in these subjects compared to females in diestrus (Fig 1.2).

Effects of GABA Receptor Antagonists on Extracellular PFC DA Levels in Male, Female, and GDX Rats

GABA-A Receptor Antagonism: Initial studies explored the effects of both the non-competitive antagonist picrotoxin and the competitive antagonist bicuculline across a range of doses (picrotoxin: 50-200μM; bicuculline: 5-50μM). Neither drug at any concentration had any obvious effects on PFC DA levels. However, only 5μM bicuculline was able to be infused for the full two-hour period without producing discernable seizure activity. Like the higher doses tested, reverse dialysis infusion of 5μM bicuculline had no measureable effects on PFC DA levels in male, female, or GDX rats (Fig 1.3). In both sexes, in GDX rats, and in females in proestrus and diestrus alike, average DA levels remained within a few percent of pre-drug baselines throughout the drug application period (Fig 1.3). There were also no obvious effects on PFC DA levels following drug offset (Fig 1.3). A two-way ANOVA (repeated measures) also identified no significant main effects of sex or of drug treatment on PFC DA level, and no significant interactions were identified between these two.

GABA-B Receptor Antagonism: The effects of reverse dialysis infusion of the GABA-B antagonist CGP52432 were tested across a range of concentrations. Below 40 μM, infusion of CGP52432 had no effects on PFC DA level in males or in females in diestrus, proestrus, or estrus at the time of drug challenge (Fig 1.4A). When infused at concentrations of 70 μM and higher, CGP52432 produced seizures in all groups. However, when infused at 50 μM or at 60 μM, CGP52432 had clear effects on PFC DA levels. These effects were quantitatively similar for the two drug concentrations, but were markedly different in males verses females (Fig 1.4A). Thus, in male rats, roughly 30 min after drug onset (50 μM), DA concentrations rose sharply to a peak that was roughly 2 times higher than baseline. However, this peak receded within the next 15 min back to levels that were approximately 60% above baseline. From there, DA levels continued to decline and were at or near pre-drug baseline levels before the end of the drug application period (Fig 1.4B). Once drug was removed, DA levels remained at pre-drug baseline levels (Fig 1.4B). The effects of CGP52432 in female rats were similar but were much larger overall (Fig 1.4B). Thus, 30 min after drug application, DA levels rose sharply to a large peak that was roughly 5-fold higher than baseline. Within the next 15 min, DA levels dropped, but only to levels that were roughly 3 times higher than baseline (Fig 1.4B). Dopamine levels continued to be elevated but underwent a slow, modest decrement for the remainder of the drug application period (Fig 1.4B). After drug removal, DA levels rapidly returned to baseline (15-30 min, Fig 1.4B). Studies were next extended to GDX, GDX-TP and GDX-E groups. In GDX rats, 15 min after drug application, DA levels began to rise and continued to do so until drug removal, increasing nearly 9-fold (Fig 1.4B). Following drug removal, DA levels returned to

baseline within 30 min (Fig 1.4B). Within 15 min of drug application, DA levels began to increase in GDX-E rats at the same rate as those of GDX rats (Fig 1.4B). However, after 30 min of drug application, DA levels began to steadily decrease, returning to baseline levels before drug removal (Fig 1.4B). Finally, similar to intact males, GDX-TP rats showed increases in DA levels following 30 min of drug application, reaching a peak of 2-fold before steadily returning to baseline (Fig 1.4B). An initial two-way ANOVA (repeated measures, data collected using 50 μM CGP52432) identified significant main effects of group ($F_{1,24}$=3.255, p = 0.029), significant main effects of drug treatment/time ($F_{2.654,63.69}$=2.654, p < 0.0001) and significant interactions between the two ($F_{10.615,63.69}$=10.615, p = 0.03) on PFC DA level. Separate within group, one-way ANOVAs (repeated measures) also revealed significant main effects of time on PFC DA level in all groups ($F_{15,75}$=2.446-9.215, p ≤ 0.001-0.008). Post hoc comparisons showed that in males, only peak DA concentrations (30 min of drug administration) were significantly higher than baseline (p = 0.008), that in females DA levels were significantly to near significantly higher than baseline until drug removal (p ≤ 0.001-0.079), in GDX rats, DA levels were significantly to near significantly higher than baseline until drug removal (p ≤ 0.001-0.088), in GDX-TP rats, DA was higher than baseline for 30-45 min after drug administration (p = 0.001-0.005), and in GDX-E rats DA was significantly higher than baseline for 30-75 min of drug infusion (p ≤ 0.001-0.005). Finally, the effects of CGP52432 on extracellular PFC DA were significantly to near significantly greater in females than in males for most of the drug application period (p = 0.0002-0.09, Fig 1.4B), significantly to near significantly greater in GDX rats compared to males for most of the drug application period (p = 0.005-0.097), and

significantly greater in GDX-E rats compared to male rats at the 30 min drug application time-point ($p = 0.035$).

Effects of Glutamate Receptor Antagonism on Extracellular PFC DA Levels in Male and Female Rats

AMPA Receptor Antagonism: The effects of the AMPA antagonist NBQX on PFC DA levels was evaluated across a narrow range of concentrations, as below 100 μM, NBQX had no discernable effects on PFC DA levels and at higher concentrations above 150 μM the drug began to fall out of solution and interfere with the HPLC analysis. However, infusion of the AMPA antagonist NBQX at 150 μM reliably decreased extracellular PFC DA in male and female rats to similar extents and according to similar timelines (Fig 1.5). Thus, in males and in females in either diestrus or proestrus (dashed lines Fig 1.5) at the time of testing, basal DA levels began to fall within 15 min of drug application, and dropped to maximally depressed extracellular DA concentrations that were roughly 30% below baseline within 45 min (Fig 1.5). Dopamine levels remained depressed for the remainder of the drug application period and upon drug removal rose back to pre-drug concentrations within 45-60 min (Fig 1.5). A two-way ANOVA (repeated measures) identified significant main effects of NBQX on PFC DA level ($F_{14,112}$=11.62, $p < 0.0001$) but no significant main effects of sex and no significant interactions between sex and drug treatment on PFC DA level. Subsequent one-way ANOVAs revealed significant main effects of time ($F_{9,63}$=6.245-8.479, $p < 0.0001$) with DA levels of males and females dropping to values that were significantly

lower than baseline within 45 min of drug onset and remaining significantly lower than baseline until the time of drug removal ($p \leq 0.001$-0.048).

NMDA Receptor Antagonism: The NMDA antagonist APV was infused via reverse dialysis into the medial PFC at concentrations of ranging from 100 to 670 μM. Prefrontal DA levels were unaffected by infusions of 160 μM or below in males and by infusions of 100 μM in females (Fig 1.6A). In male rats, intermediate doses of APV (330-500 μM) increased PFC DA levels by 30-50% in a concentration-dependent manner whereas in females, 160 μM-500 μM APV decreased PFC DA levels by 40-50%, also in a concentration dependent manner (Fig 1.6A). For both sexes, the respective effects of 500 and 670 μM APV were similar (Fig 1.6A). During a 2-hour drug infusion protocol using 500 μM APV, in males, PFC DA levels began to rise within 15 min of drug onset (Fig 1.6B). By 60 min PFC DA levels reached peak concentrations of 30-40% above baseline that were sustained until drug offset (Fig 1.6B). After APV was removed from the infusion, PFC DA levels returned to pre-drug levels in approximately 30 min (Fig 1.6B). In females in either diestrus or proestrus at the time of drug challenge, PFC DA levels were slightly more sluggish in responding. However, 15-30 min after drug infusion, PFC DA concentrations dropped to levels of 30-40% below baseline that were sustained for the remainder of the drug application period (Fig 1.6B). After drug offset, PFC DA levels rose back to baseline within 30 min (Fig 1.6B). An initial two-way ANOVA (repeated measures) identified significant main effects of sex ($F_{1,8}$=16.05, p = 0.0039) and significant interactions between sex and APV treatment ($F_{14,112}$=7.184, p < 0.0001) on PFC DA levels. Separate within-sex one-way ANOVAs

revealed significant main effects of time on PFC DA levels in males and females ($F_{9,79}$=3.381-6.387, $p \leq$ 0.0001-0.002). Post hoc comparisons showed that in males, APV induced DA levels that were significantly higher than baseline from 60 min of drug infusion until drug removal (p = 0.001-0.015), that in females, APV decreased DA to levels that were significantly to near significantly lower than baseline from 60 min of drug infusion to drug offset (p = 0.040-0.087) and that the effects of APV effects on PFC DA levels were significantly different in males verses females from 45 min after drug infusion until drug removal ($p \leq$ 0.0001-0.0032).

Effects of Dual GABA Receptor /NMDA Receptor Antagonism on Extracellular PFC DA Levels in Male, Female Rats, GDX and GDX-hormone replaced rats

A dual drug reverse dialysis infusion challenge was carried out in which CGP52432 (50μM) was introduced 45 min prior to adding 500μM of APV to the dialysate, after which time both drugs were co-infused for an additional 90 min (Fig 1.7). In male rats, the prior infusion of CGP52432 blocked the expected DA-stimulating actions of APV (Fig 1.7A). Thus, 30 min after CGP52432 infusion, DA levels transiently peaked at levels that were about two-fold higher than baseline. Within 15 min, however, DA levels dropped back to levels that were roughly 60% higher than baseline and continued to fall thereafter to near pre-drug baseline levels, despite the addition of APV to the infusion (Fig 1.7A). When both drugs were removed, DA levels returned to pre-drug concentrations within about 15 min (Fig 1.7A). In female rats in diestrus or proestrus at the time of challenge, the CGP52432/APV co-infusion protocol had no obvious effects on APV's suppression of PFC DA levels (Fig 1.7B). Thus, CGP52432

administration induced an expected peak in DA level. However, rather than remaining above baseline, when APV co-infusion commenced, PFC DA levels dropped to levels of 30-40% below baseline (Fig 1.7B). Dopamine levels remained depressed until the co-infusion of both drugs stopped; after this, it took roughly 60 min for PFC DA levels to return to baseline (Fig 1.7B). Similar to females, GDX rats showed expected, approximately 4-fold increases in PFC DA levels following CGP52432 administration (Fig 1.7C). However, following APV co-infusion, DA levels dropped to nearly 40% below baseline, which was sustained until drug removal (Fig 1.7C). Rats in the GDX-E group showed similar responses to drug infusion. Following application of CGP52432, DA levels increased nearly 2-fold then proceeded to drop approximately 40% below baseline following APV co-infusion (Fig 1.7D). The depressed DA levels were sustained until drug removal wherein they returned to near baseline levels (Fig 1.7D). Finally, GDX-TP rats showed an over 2-fold increase in PFC DA levels following application of CGP52432, which began to fall after 30 min and return to baseline levels despite co-infusion of APV (Fig. 1.7E). An initial two-way ANOVA (repeated measures) identified significant main effects of group ($F_{1,24}$=4.25, p = 0.010), significant main effects of drug treatment ($F_{4.427,106.257}$=7.931, p < 0.001) and significant interactions between group and drug treatment ($F_{17.709,106.257}$=1.996, p = 0.016) on PFC DA levels. Within-sex, one-way ANOVAs further revealed significant main effects of time/drug on PFC DA levels in all groups except GDX-TP ($F_{14,56}$=3.509-11.000, $p \leq 0.001$). Allowed post-hoc analyses showed that in males, only the peaks in DA level that followed 30 min of CGP52432 application were significantly higher than baseline (p < 0.001). In females, initial, CGP52432-induced peaks in DA level were also significantly higher than baseline

(p = 0.065); following co-infusion of APV, within 75 min, DA levels dropped to levels that were nearly significantly lower than baseline (p = 0.077). In GDX rats, only time points 30-45 min into CGP52432 application were significantly higher than baseline ($p \leq 0.001$-0.006). In GDX-E rats, time points 30-45 min into CGP52432 application were significantly to near significantly above baseline ($p \leq 0.001$-0.078). Following APV co-infusion, DA levels dropped below baseline and approached significance in the final 30 min of drug infusion ($p = 0.064$-0.081).

DISCUSSION

The executive functions of the PFC rely on and are responsive to basal levels in extracellular DA concentration (Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Landau et al., 2009; Cools and D'Esposito, 2011). These DA levels are governed by regulatory circuits and mechanisms that continually set and re-set PFC DA tone. Not surprisingly, factors that interfere with homeostatic processes and move PFC DA levels away from optimal operational settings, e.g., stress, drug stimulation, disease (Davis et al., 1991; Deutch, 1992; Murphy et al., 1996; Zahrt et al., 1997; Goldberg et al., 2003; Arnsten and Li, 2005; Niwa et al., 2010; Dumontheil et al., 2011; Cervenka et al., 2012) negatively impact executive function. This study asked whether the sex differences that also characterize DA-dependent executive functions and executive dysfunctions related to hyper- or hypodopaminaergia (Ott et al., 1996; Overman et al., 1996; Szymanski et al., 1996; Leung and Chue, 2000; Goldstein et al., 2002; Petry et al., 2002; Canuso and Pandina, 2007; Usall et al., 2007; Miller and Cronin-Golomb, 2010; Lai et al., 2012; Feinstein and Kritzer, 2013), might be due to

dimorphisms in the homeostatic mechanisms that regulate PFC DA levels. Recent *in vivo* microdialysis, reverse dialysis drug application studies showed that the intracortical AMPA and NMDA receptor-mediated GLU mechanisms that are well known to tonically regulate PFC DA levels (Feenstra et al., 1995; Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Aubele and Kritzer, 2012) are sensitive to circulating gonadal steroids in adult male rats (Aubele and Kritzer, 2012). Here similar approaches were used to compare the effects of reverse dialysis infusion of selective GABA-A, GABA-B, AMPA, and NMDA receptor antagonists on extracellular PFC DA levels in male, female , GDX, GDX-E, and GDX-TP rats. These studies showed that the effects of GABA-A and AMPA antagonists on PFC DA levels were indistinguishable across sex and hormone treatment group. Thus, GABA-A antagonism had no measureable impact on PFC DA levels in male, female, or GDX rats and AMPA antagonism, which has been repeatedly shown to depress extracellular PFC DA levels in males (Jedema and Moghddam, 1996; Jin, 1997; Takahata and Moghaddam, 1998; Aubele and Kritzer, 2012), and previously shown to have markedly reduced effects in GDX and GDX-E, but not GDX-TP rats (Aubele T and MF Kritzer 2012) was found to have qualitatively and quantitatively similar effects in females and males. In contrast, infusion of GABA-B and NMDA antagonists had markedly different effects on basal PFC DA levels across sex and hormone treatment groups. First, in contrast to previous studies (Harte and O'Connor, 2005; Balla et al., 2009), GABA-B antagonism was found to have transient, DA-potentiating actions in males. Further, while GABA-B blockade was also DA-potentiating in females, the increases induced in PFC DA levels were several-fold larger and much longer lasting than in males. Additionally, GABA-B

antagonism caused steady, continuous increases in PFC DA levels of GDX rats, which were also several-fold larger than in males while in GDX-E rats, GABA-B antagonism led to transient DA increases that were similar to, albeit slightly larger than, those of intact males. In GDX-TP rats, effects of GABA-B antagonism were indistinguishable from those observed in intact male rats. And finally, while NMDA antagonism increased extracellular DA in males as expected (Feenstra et al., 1995; Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Aubele and Kritzer, 2012), its infusion in females significantly decreased PFC DA levels as it was previously shown to do in GDX and GDX-E but not GDX-TP rats (Aubele and Kritzer, 2012). These latter findings suggested that long held views of intracortical NMDA-mediated influences as being levied on PFC interneurons (Homayoun and Moghaddam, 2007b; Yonezawa et al., 1998) describe DA regulation in the male but not the female PFC. Instead, our data suggest that NMDA antagonism in females acts independently of interneurons in regulating PFC DA levels. This was borne out in co-infusion studies where antagonism of intra-PFC GABAergic signaling blocked the DA-potentiating effects of the NMDA antagonist APV in male and GDX-TP rats, but had no effect on APV's DA-depressing actions in female, GDX, and GDX-E rats. In the sections below, these findings are considered further in relation to the extant literature describing amino acid transmitter regulation of PFC DA tone. Findings are also discussed in contexts of the known impact of sex and sex hormones on cortical amino acid transmitter systems and are incorporated into sex-specific models of the seemingly disparate ways in which the male and the female PFC sustains basal DA levels that are remarkably similar to each other in terms of absolute concentration.

Tonic GABAergic Regulation of PFC DA Levels: Prefrontal DA-regulating GABAergic systems were investigated using intracortical reverse dialysis infusion of the GABA-A antagonists picrotoxin and bicuculline, and the GABA-B antagonist CPG52432 across a range of concentrations. This was performed in part to identify conditions where drug infusion did not produce seizures. Although sex differences and/or hormone effects have been described in susceptibility to ictal activity including that induced by bicuculline and picrotoxin (Pericic et al., 1996; Bujas et al., 1997; Frye, 2008, 2010) we found that seizure-producing thresholds for all three drugs were similar in males, females, and hormone-manipulated rats. For all three drugs as well, these thresholds were substantially lower and often half of the drug concentrations used in previous *in vivo* microdialysis/reverse dialysis studies (Santiago et al., 1993; Harte and O'Connor, 2005; Fallon et al., 2007; Balla et al., 2009). This may be related to the use of Ringer's solution (prior studies) verses artificial CSF (present study) to dissolve and deliver drugs, as most other parameters of experimental design were similar. Regardless, it is important that the negative results obtained in the current study using both competitive (bicuculline) and non-competitive (picrotoxin) GABA-A antagonists corroborate those obtained from the prior studies using either higher (Santiago et al., 1993; Harte and O'Connor, 2005; Fallon et al., 2007; Balla et al., 2009) or lower (0.1 μM) concentrations than used here (Harte and O'Connor, 2004). Thus, with the exception of studies in which the non-transportable DA uptake blocker nomifensine was co-infused with the GABA-A antagonist picrotoxin (Santiago et al., 1993), the consensus is for no discernable effects of intraPFC infusion of either competitive or non-competitive

GABA-A antagonists on basal PFC DA level or on GLU overflow in the ventral tegmentum (Harte and O'Connor, 2005; Fallon et al., 2007).

Like GABA-A, previous studies also found no effects of GABA-B antagonism on PFC DA or ventral midbrain GLU levels, again using drugs including CGP52432 at concentrations that were up to two-fold higher than those used here (Takahata and Moghaddam, 1998; Harte and O'Connor, 2005; Balla et al., 2009). However, our data suggest a tonic GABA-B-mediated suppression of PFC DA levels in male AND female. This discrepancy may be related to the fact that in our hands, GABA-B blockade produced surges in PFC DA concentration that in males appeared and returned to near-baseline levels within about 30 min. Thus it is possible that the 30 min sampling periods and the use of male subjects exclusively in each of the prior studies (Harte and O'Connor, 2005; Fallon et al., 2007; Balla et al., 2009) served to average out and obscure these transient GABA-B responses.

In female rats we found that CGP52432 also produced spikes in PFC DA levels, albeit ones that were significantly larger and that took significantly longer to return to baseline than those produced in males. These results were strikingly similar to those in GDX rats; compared to intact males, CGP52432 also produced significantly larger, long-lasting PFC DA increases. This reinforces ideas that low levels of androgens are in part what shapes size of DA surge that makes up part of the sex difference observed in GABA-B responses. However, there were also differences in resetting mechanisms. Thus, it was evident in male, female, but not GDX rats that the surges in PFC DA level induced by CGP52432 infusion triggered some sort of GABA-B-independent, rectifying response(s) that brought DA concentrations back down to or nearer to baseline levels.

That these were also seen in both in GDX-E and GDX-TP rats suggests that whatever is being engaged, it may be subject to estrogen regulation. An attractive candidate for this resetting mechanism in the male brain may be DA activation of intracortical DA D2 receptors. Although DA affects PFC excitability in complex, concentration and receptor-subtype dependent ways (Seamans and Yang, 2004; Tseng and O'Donnell, 2004; Williams and Castner, 2006), in the male brain DA D2 agonists have been shown to activate fast-spiking PFC interneurons (Tseng and O'Donnell, 2004, 2007a, b), to stimulate local GABA overflow (Retaux et al., 1991; Grobin and Deutch, 1998), to mediate DA potentiation of GLU-stimulated GABA overflow (Del Arco and Mora, 2000) and to inhibit PFC pyramidal cell activity in part via GABA-A sensitive means (Tseng and O'Donnell, 2007b). These and other D2-mediated actions are concentration-dependent and typically emerge only under conditions of elevated to supranormal DA levels (Zheng et al., 1999; Del Arco and Mora, 2000). This sort of DA driven, GABA-A sensitive, GABA-B independent inhibition fits criteria for mechanisms capable of self-limiting the spikes in DA level that we observed to be induced by intra-PFC infusion of CGP52432. Moreover, DA D2 stimulation's net inhibitory actions have been shown, again to date in the male brain only, to emerge after adolescence (O'Donnell, 2010) suggesting links or sensitivity to circulating gonadal steroids. Given the importance of D2 signaling to PFC network operations (Seamans et al., 2001; Xu et al., 2009; Gruber et al., 2010) and to clinical therapeutics (Laruelle et al., 2005; Masana et al., 2012), it will be important to verify this potential involvement in DA re-setting functions and determine whether and how biological sex and/or sex hormones impact these processes.

Tonic GLU Regulation of PFC DA Levels: Prefrontal DA-regulating GLU systems were investigated here in male and female rats using reverse dialysis intraPFC infusion of the AMPA antagonist NBQX and the NMDA antagonist APV. These along with other antagonists of AMPA and NMDA GLU receptors have been extensively used in *in vivo* microdialysis studies of PFC DA regulation in rats. Carried out exclusively in males, these studies along with data from male subjects of the present study all show that in this sex, AMPA-antagonism in the PFC depresses extracellular DA levels (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Del Arco and Mora, 1999; Wu et al., 2002; Aubele and Kritzer, 2012) and intracortical NMDA antagonism potentiates PFC DA levels (Feenstra et al., 1995; Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Del Arco and Mora, 1999; Aubele and Kritzer, 2012). While these data have been interpreted as evidence for a tonic AMPA receptor-mediated drive and a tonic NMDA receptor-mediated suppression over mesocortical DA systems and DA overflow in the PFC, our studies suggest that this is only half correct for the female brain. Thus, while intraPFC infusion of NBQX dose-dependently decreased extracellular PFC DA levels similarly in both sexes, APV dose-dependently decreased extracellular PFC DA levels in females -- the opposite of its DA potentiating actions in males and similar to what has been observed previously in GDX and GDX-E but not GDX-TP rats (Aubele and Kritzer, 2012). In sum, while in males intracortical NMDA and AMPA receptor-mediated actions exert opposing, functionally balancing influences over the activity of mesocortical DA afferents, in female, GDX, and GDX-E rats, both receptor subtypes appear to tonically drive midbrain DA systems. That the effects of GDX on APV were

attenuated by supplementing GDX rats with testosterone propionate but not estradiol suggests that diminished androgen is responsible for the anomalous drug effects observed (Aubele and Kritzer, 2012). It is thus tempting to speculate that the chronically low levels of androgen experienced by the female brain likewise contribute to their sex-specific modes of NMDA-mediated PFC DA homeostasis.

Relevance for working model. Similar to what has been posited for GDX male rats (Aubele and Kritzer, 2012), the impact of APV on PFC DA levels in female rats suggests an NMDA-mediated stimulation that directly excites PFC pyramidal cells, including those projecting to the ventral midbrain. This is fundamentally different from current, seemingly male-specific evidence of NMDA receptor-mediated effects on PFC DA homeostasis as being translated through GABAergic interneurons (Homayoun and Moghaddam, 2007b; Yonezawa et al., 1998). These sex-specific circuit schemas are consistent with outcomes from the present CGP52432/APV co-infusion studies wherein the effects of NMDA antagonism on PFC DA levels were found to be dependent on GABAergic signaling in males and GDX-TP rats, but independent of GABAergic inhibition in the females, GDX and GDX-E rats

The list of activational hormone effects and/or sex differences among NMDA receptors and NMDA receptor-mediated actions in rat cerebral and hippocampal cortices is considerable and continues to grow. These include effects of estrogens and androgens on pyramidal cell spine densities (Gould et al., 1990; Woolley and McEwen, 1993; Leranth et al., 2003; Leranth et al., 2004; Hajszan et al., 2008), on NMDA receptor numbers, affinities and/or subunit compositions (Smith and McMahon, 2006;

Taherianfard et al., 2012; Vedder et al., 2013), and on measures of NMDA-dependent toxicity and synaptic plasticity (Pozzo-Miller et al., 1999; Kajta et al., 2001; de Olmos et al., 2008; Smith et al., 2010). We hypothesize that the sex differences in NMDA-mediated PFC DA homeostatic mechanisms identified in this study have their origins in a differential NMDA receptor-mediated activation of VTA-projecting PFC pyramids. How this occurs is unknown but could be related to differences in genomic androgen actions as these corticofugal neurons are uniquely and heavily invested with the requisite intracellular receptive machinery in both male and female rats (Aubele and Kritzer, 2012) and findings here of attenuation of GDX by TP but not E points quite directly to androgen as the active moiety. Thus, as previously argued for GDX male rats (Aubele and Kritzer, 2012), plausible targets of this genomic activation include protein kinase C which is known to be activated by AR-dependent androgen signaling (Nguyen et al., 2009) and is capable of stimulating Ca-dependent NMDA receptor deactivation (Lu et al., 2000). However, other alternatives, including impact on NMDA receptor trafficking similar to that recently demonstrated for the neurosteroid pregnenolone (Kostakis et al., 2013) should also be considered.

Summary and Conclusions. The importance of DA regulation for executive functions of the PFC along with suspected roles for DA imbalance in the cognitive dysfunction seen in organic and preclinical disease models have led to studies using *in vivo* microdialysis and other methods in adult rats exploring the intracortical systems that regulate PFC DA levels. A large body of work sums to identify tonic intracortical AMPA-mediated stimulation of VTA-projecting PFC pyramids that drives mesoprefrontal

DA neurons and keeps PFC DA levels elevated and an opposing intracortical NMDA-mediated drive of PFC GABAergic interneurons that inhibits VTA-projecting pyramids and holds PFC DA levels in check (Feenstra et al., 1995; Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Del Arco and Mora, 1999; Wu et al., 2002; Aubele and Kritzer, 2012). Together, these mechanisms form bases for contemporary computational models of PFC function (Berridge and Robinson, 1998; Durstewitz et al., 2000; Durstewitz and Seamans, 2002; O'Donnell, 2003; Seamans and Yang, 2004) and are strongly influential in shaping etiologic thinking about mental illness and its treatment (Lewis and Moghaddam, 2006; Seeman, 2009; Javitt, 2010; Moghaddam and Javitt, 2012; Stan and Lewis, 2012). However, the foundation studies all share limitations of only examining male subjects. The present comparative studies in male, female, and hormone-manipulated male rats add new information about a previously unrecognized contribution of GABA-B receptor-mediated mechanisms to the tonic suppression of PFC DA levels and for significant sex and sex-hormone mediated differences in this and in the NMDA receptor-mediated DA homeostatic mechanisms that are at work in the male and female PFC. It may be important to consider these findings further in two contexts. The first is one of the sexually dimorphic mesoprefrontal projections wherein DAergic cells of origin are roughly two times more numerous in females than in males (Swanson, 1982; Deutch et al., 1991; Carr and Sesack, 2000b; Margolis et al., 2006; Kritzer and Creutz, 2008). The second relates to basal PFC DA concentrations that are by most accounts similar across sex (Tanila et al., 1994; Duchesne et al., 2009). From these we hypothesize that in females, potent intraPFC, DA-facilitating GLU influences, uniquely conferred by both of its major classes

of cortical ionotropic receptors, drive mesoprefrontal systems that are characterized by a doubling of constituent DA cells of origin relative to males. However, while this might be expected to potentiate DA levels in the female relative to the male PFC—and similar to what happens in GDX (Aubele and Kritzer, 2011), companion findings of a significantly more powerful GABA-B-mediated DA inhibition in the female PFC may be what maintains basal DA concentrations that are in fact similar across sex. Our findings also provide a key piece of data supporting the working model describing an androgen-driven cellular shift in the locus of NMDA influence. These sex/sex hormone-specific means of PFC DA homeostasis may also shape the sex-differences that differentiate PFC functions in humans and animals. They might also shed new light on the neurobiology relevant to females' disproportionate vulnerability to stress, depression, PTSD and other anxiety disorders associated with PFC hyperdopaminergia, and their relative protection from the cognitive deficits associated with PFC hypodopaminergia in disorders such as schizophrenia. This is also a critical first step in upholding the working model of my dissertation which will be further tested in the next chapter by exploring its predictive power for cell firing/activity in and among homeostatic cells and circuits.

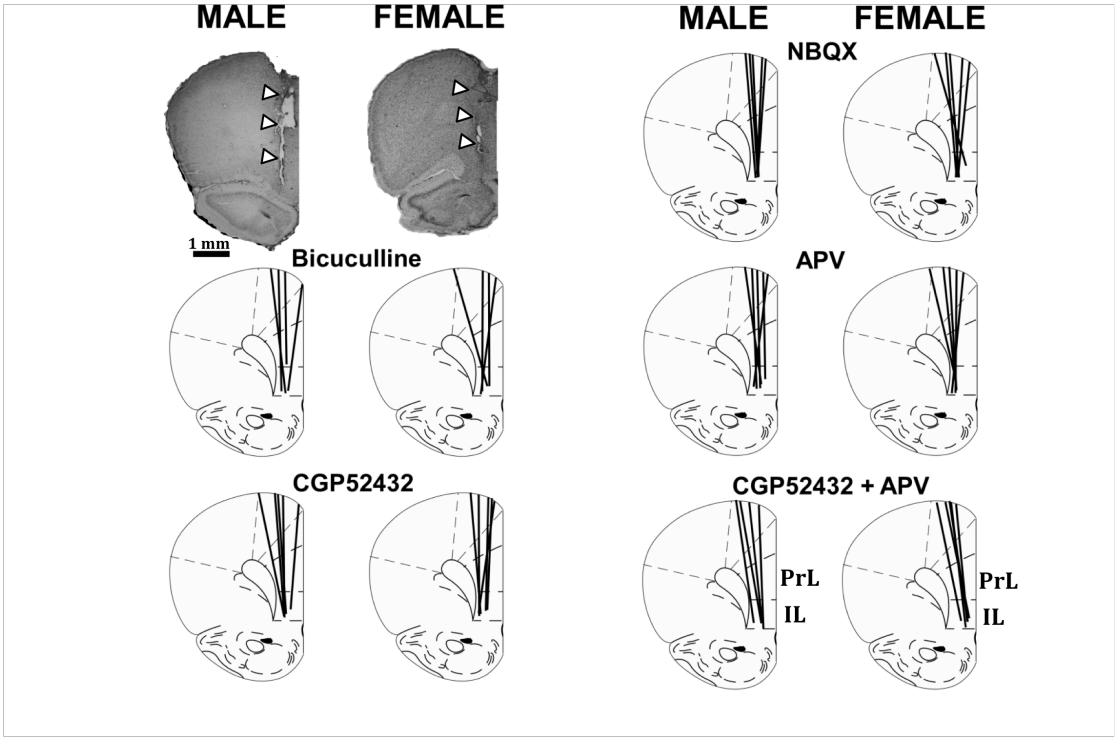

Figure 1.1. Representative low power photomicrographs (top left) and line drawings (rows below) showing the locations of microdialysis probes tracks in relation to cytoarchitecture of the prefrontal cortex in male (left columns) and female (right columns) rats. Tissue sections are counterstained with cresyl violet and visible damage from the probe tracks is identified by white triangles. Line drawings and their cytoarchitectonic boundaries are as per (Paxinos and Watson, 1998); the locations of microdialysis probe tracks are depicted as black lines. The antagonist drug study is identified above line drawing pairs. Scale bar = 1 mm. Abbreviations: IL, infralimbic cortex; PrL, prelimbic cortex.

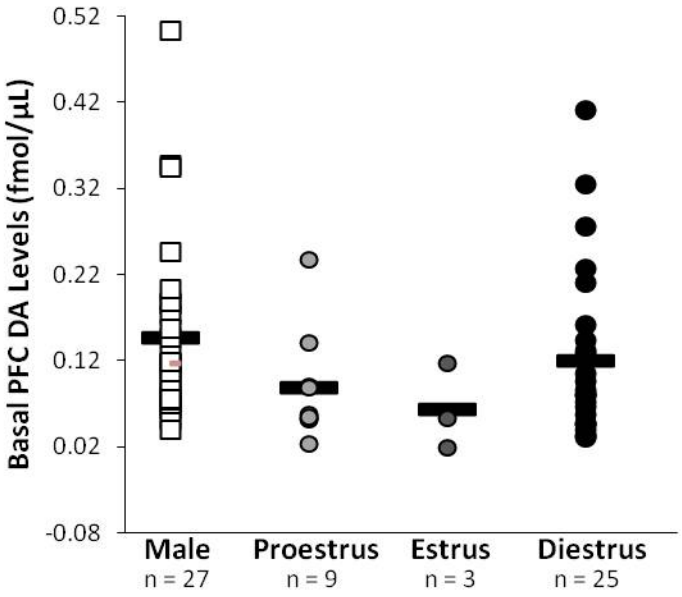

Figure 1.2. Scatter plots showing basal dopamine level concentrations (fmol/µL) measured from all individual subjects included in this study. Group means are depicted by horizontal black bars. Males are shown in white squares; females are shown in circles and separated by estrous cycle stage – proestrus (light gray circles), estrus (dark gray circles), and diestrus (black circles). No significant differences were found in basal dopamine levels among any of these groups or in comparisons in which males were compared to a single combined pool of all female subjects.

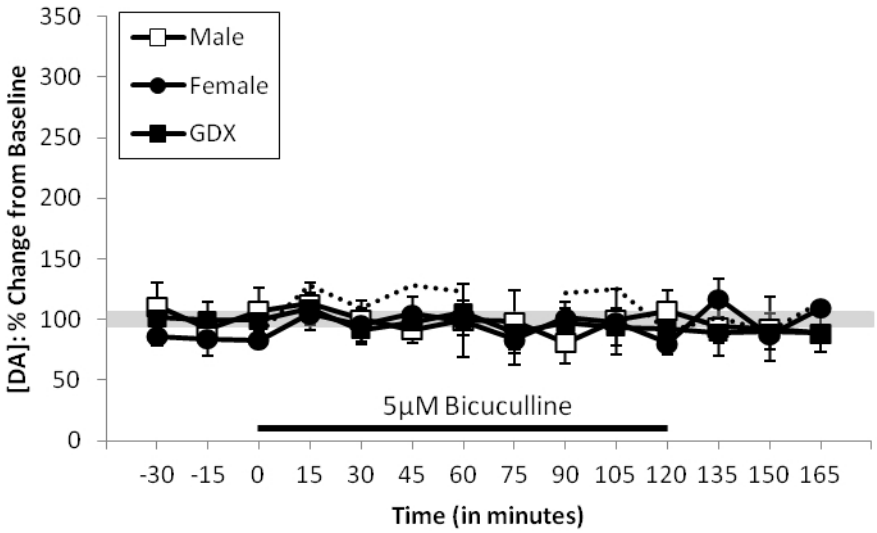

Figure 1.3. Timeline/line graphs showing the effects of reverse dialysis application of 5 μM bicuculline on extracellular prefrontal dopamine (DA) levels, expressed as mean percent changes from baseline (± standard error of the mean) in male (white squares) and female (black circles) rats, or GDX (black squares). The solid black line beneath the line graphs marks the drug infusion period. Bicuculline did not have any significant effects on DA concentration in either males, females, or GDX rats. The timeline from the single female subject in proestrus at the time of the experiment is also shown in the black dashed line; there are no apparent differences in the responses of this animal compared to the female group mean.

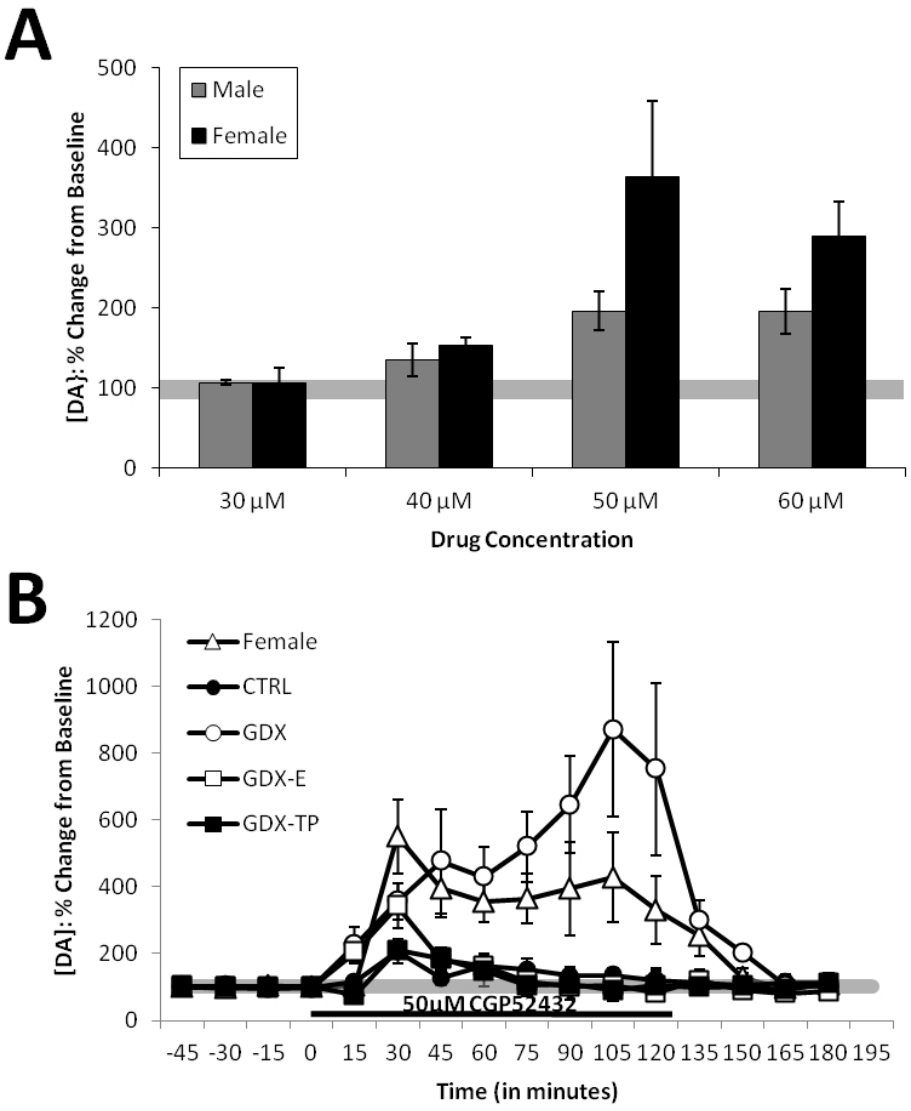

Figure 1.4. Bar graphs (A) showing the maximum effects of CGP52432 on extracellular prefrontal dopamine (DA) at different drug concentrations. Timeline/line graphs (B) showing the effects of reverse dialysis application effects of CGP52432 on extracellular prefrontal DA levels expressed as mean percent change from baseline (± standard error of the mean). The solid black line beneath the line graph in B marks the drug infusion period. (A) In both sexes, the lowest concentration of CGP52432 (30 μM) had no effect on extracellular DA levels and maximal, seizure-free effects were reached at the 50 μM concentration. (B) Within 30 min of application, CGP52432 (50 μM) increased in prefrontal DA levels in all groups but much more so in females (white triangles) and GDX rats (white circles), which remained elevated throughout drug application. DA levels in male (black circles), GDX-E (white squares), and GDX-TP (black squares) rats returned to baseline within the drug application period.

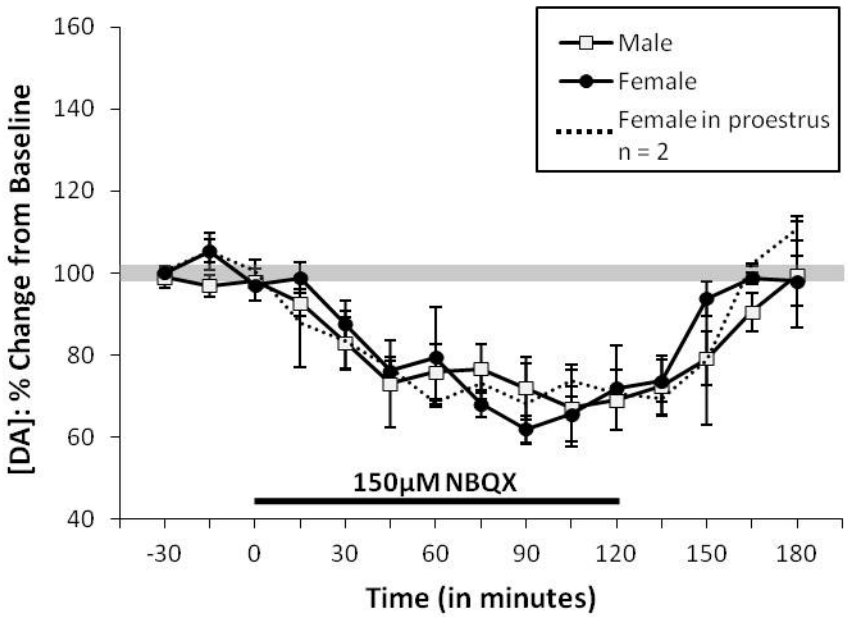

Figure 1.5. Timeline/line graphs showing the effects of reverse dialysis application of NBQX on extracellular prefrontal DA levels expressed as mean percent change from baseline (± standard error of the mean) in male (white squares) and female (black circles) rats. The solid black line beneath the line graphs marks the drug infusion period. In both sexes, NBQX application decreased in prefrontal DA levels within 30 min. The data from two females in proestrus at the time of the experiment are graphed in the black dashed line; there are no apparent differences in the responses of these subjects compared to the female group mean.

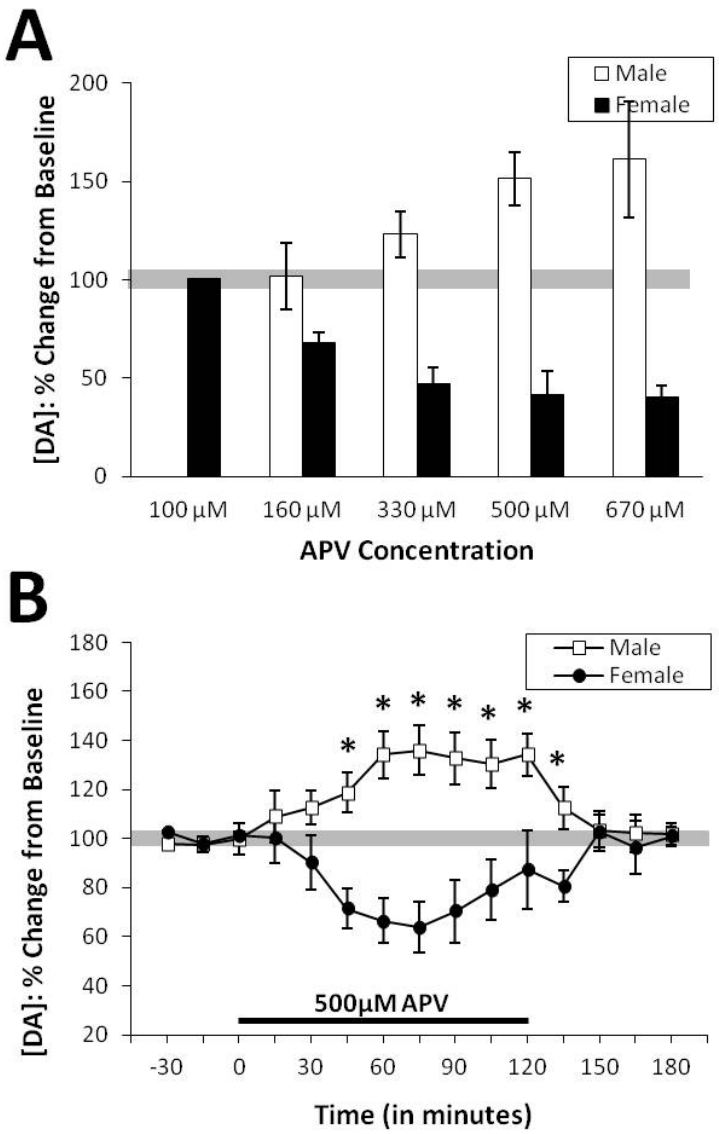

Figure 1.6. Bar graphs (A) showing opposite, dose-dependent effects of APV on extracellular prefrontal dopamine (DA) in male (white bars) and female (black bars) rats across a range of drug concentrations. APV at 100 μM and 160 μM had no effects on prefrontal DA levels in females (black bars) or males (grey bars), respectively. At higher doses, APV progressively increased prefrontal DA levels in males, and decreased prefrontal DA levels in females. In both sexes, maximum drug effects on extracellular DA levels were achieved at 500 μM APV. (B) Timeline/line graphs showing the effects of 500 μM APV on extracellular prefrontal DA levels expressed as mean percent change from baseline (± standard error of the mean) in male (white squares) and female (black circles) rats. The solid black line beneath the line graphs in B marks the drug infusion period. Infusion of 500 μM APV rapidly increased DA levels in males but decreased DA levels in females. Asterisks identify the time points where DA levels in males and females were significantly different from each other ($p < 0.05$).

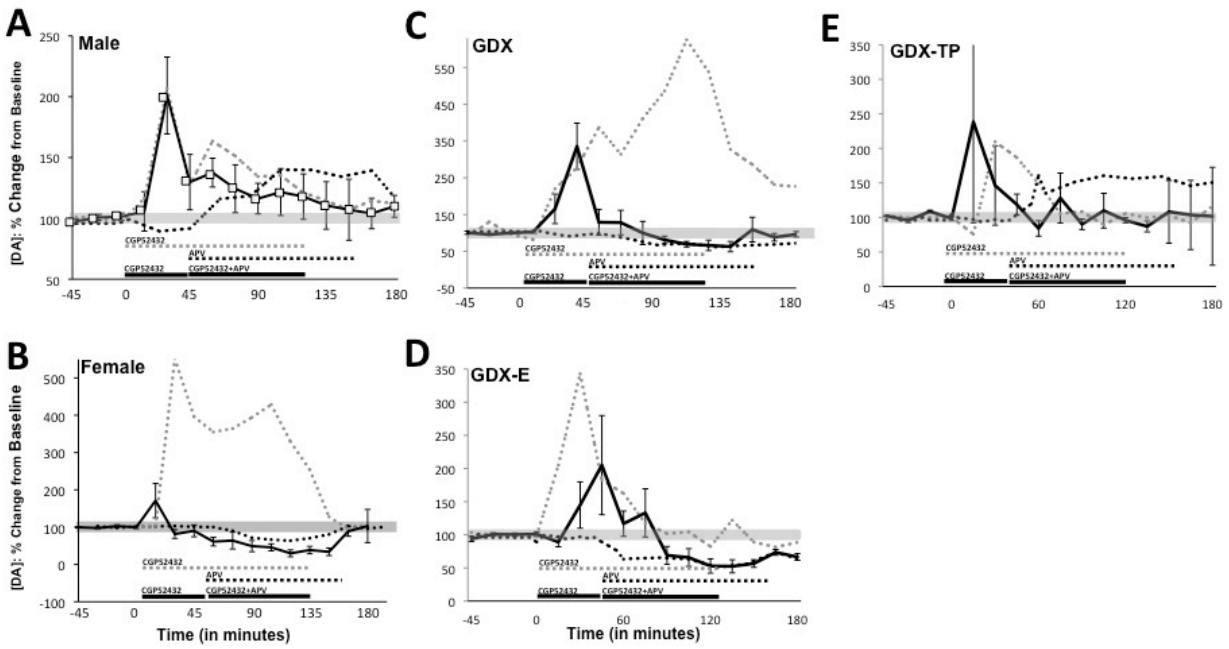

Figure 1.7. Timelines/line graphs showing the effects of reverse dialysis infusion of CGP52432 followed by co-infusion of CGP52432 and APV (black line) on extracellular prefrontal DA levels expressed as mean percent change from baseline (± standard error of the mean) in (A) male and (B) female rats. For reference, the effects of infusion of CGP52432 (light gray dashed line), and APV (medium gray dashed line) alone are also graphed. (A) In males, initial infusion of CGP52432 alone increased extracellular DA levels as expected (light gray dashed line) while the subsequent addition of APV to the infusate failed to increase DA levels as would be expected from APV infusion alone (medium gray dashed line). Rather, DA levels continued to decline as in conditions of CGP52432 infusion alone (light gray dashed line). (B) In females, initial infusion of CGP52432 alone also produced expected increases in extracellular DA levels (light gray dashed line). However, the subsequent addition of APV to the infusate decreased DA to depressed levels that were similar to those evoked by APV alone (medium gray dashed line) and that were markedly different from the elevated PFC DA levels associated with CGP52432 infusion (light gray dashed lines). (C) GDX rats similarly showed reductions in PFC DA levels following co-infusion of CPG52432 and APV as did GDX-E rats (D). However, like intact males, the effects of APV on DA levels in GDX-TP rats (E) were blocked by CGP52432 co-infusion.

Chapter II

Sex and gonadal hormone effects on cell firing rates and bursting activity of ventral tegmental area-projecting prefrontal pyramidal cells and dopaminergic VTA cells.

The overall objectives of my dissertation are to explore/define the neurobiological basis for the effects of sex and sex hormones on the DA-dependent executive functions of the PFC. Sex differences are well known to exist in normal PFC function and in PFC dysfunction in disease (Goldman et al., 1974; Roof et al., 1993; Ott et al., 1996; Overman et al., 1996; Lacreuse et al., 1999; Leung and Chue, 2000; Petry et al., 2002; Jentsch and Taylor, 2003; Bayless et al., 2012; Lai et al., 2012). These processes are highly dependent on optimal PFC DA tone and on abnormalities in PFC DA levels, respectively (Davis et al., 1991; Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Goldberg et al., 2003). The overall hypothesis of my work is that gonadal hormones regulate PFC DA levels in the male and female brain by modulating the intracortical amino acid transmitter systems and circuits that tonically maintain DA homeostasis in the PFC. In Chapter I, the receptor subtype-selective GLUergic and GABAergic mechanisms that regulate PFC DA levels were compared across sex differences and among CTRL, GDX, GDX-E, and GDX-TP male rats to confirm hypotheses about roles for circulating androgens in shifting the sphere of NMDA-R-mediated GLU regulation of PFC DA levels between inhibitory interneurons and PFC pyramidal cells. Previous work in gonadally intact male rats, including work showing that intraPFC antagonism of NMDA receptors results in an elevation of PFC DA levels

(Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Aubele and Kritzer, 2012), has established consensus views for DA-regulating NMDA effects as occurring through GABAergic interneurons in the PFC (Homayoun and Moghaddam, 2007a). However, as reported in Chapter I, NMDA receptor antagonism has the opposite effects not only in GDX rats (Aubele and Kritzer, 2012), but also in females suggesting that hormones, likely androgens specifically, suppress the NMDA sensitivity of PFC pyramidal cells -- including those projecting to the VTA that control cell firing in the VTA and thus DA release in the PFC (Lodge, 2011). Chapter I also presented further support for the working model, i.e. that NMDA inhibits PFC DA levels by stimulating GABAergic interneurons in CTRL and GDX-TP males, (subjects where circulating androgen levels are high) but increased PFC DA levels in GDX, GDX-E, and females (subjects where circulating androgen levels are low), by exciting VTA-projecting PFC pyramidal cells. By using dual drug challenge, it was shown that in CTRL and GDX-TP rats, NMDA effects on PFC DA levels required GABA signaling whereas in GDX, GDX-E, and females, NMDA effects on PFC DA levels occur independently of GABAergic interneurons. This adds significantly to previous support for the model's proposed centrality of hormone-induced changes as targeting VTA-projecting PFC pyramidal cells rather than elsewhere among the DA-regulating circuits based on immunohistological studies showing significant intracellular androgen receptor (AR) enrichment of these cells (Aubele and Kritzer, 2012).

Thus, evidence is mounting in support of an NMDAR- heightened excitatory drive over VTA DAergic cells being induced by chronically low androgen levels as explaining the significantly elevated basal PFC DA levels found in GDX and GDX-E compared to

CTRL and GDX-TP male rats (Aubele and Kritzer, 2011). As explained in Chapter I, though DA levels are similar in male and female rats, the comparatively greater NMDA-sensitivity of PFC pyramidal cells in females proposed in the working model is still likely and possibly offset by the heightened GABA-B mediated inhibition of the PFC that was also found for this sex. In this chapter, electrophysiology is used to test predictions that this model holds for cell activity in the PFC and the VTA in CTRL, GDX, GDX-E, GDX-TP, and female rats.

While in vitro electrophysiological recordings in PFC slices from male rats have demonstrated tonic NMDA receptor currents in both pyramidal cells and interneurons (Povysheva and Johnson, 2012), the body of *in vivo* microdialysis and electrophysiological work demonstrates inhibitory effects of NMDA on PFC pyramidal cells and PFC DA levels that are presumed to occur via activation of interneurons (Homayoun and Moghaddam, 2007a). For example, in vitro studies have shown that stimulus-evoked excitatory post-synaptic potentials are stronger and elicited by lower activating currents in fast-spiking PFC interneurons than in pyramidal cells (Povysheva et al., 2006) and extracellular PFC recordings show that following systemic injection of an NMDA receptor antagonist, interneuron firing rates decrease while pyramidal cell firing rates show a delayed increase (Homayoun and Moghaddam, 2007a). In essence, the sum of work, carried out to date only in the male PFC indicates that NMDAR agonists excite PFC interneurons and disynaptically inhibit PFC pyramids, while antagonists result in disinhibition of PFC pyramids (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Aubele and Kritzer, 2012). Incorporating these data into the working model predicts that the elevated NMDA-

mediated influence suspected for the VTA-projecting PFC pyramidal cells in GDX and GDX-E rats would lead to mean PFC pyramidal cell firing rates that are higher in these groups than in CTRL and GDX-TP rats. By also incorporating findings from Chapter I for enhanced GABA-B mediated inhibition in the PFC of female rats, I predict that pyramidal cell firing rates in these rats will be similar or even below those of CTRL and GDX-TP males. In addition to mean firing rate, there are also specific predictions for bursting. Based on slice studies showing that NMDA antagonists reduce this mode of firing in PFC pyramids (Burgos-Robles et al., 2007), I predict to observe an increased number of spikes per burst as well as increased percentage of spikes in bursts in GDX and GDX-E rats compared to CTRL, GDX-TP, and female rats in accordance with the heightened NMDA-mediated influence of the PFC pyramidal cells suspected for these animals.

PFC-originating pyramidal cell inputs to the VTA make monosynaptic connections onto DAergic and GABAergic cells in the ventral midbrain (Carr and Sesack, 2000a). Electrical activation of these descending pathways has been shown to drive DA and non–DA cells in the VTA and stimulate DA and GLU efflux (Murase et al., 1993; Karreman and Moghaddam, 1996; Harte and O'Connor, 2005; Lodge, 2011). Further, bursting activity in VTA-projecting pyramidal cells has been shown to also initiate bursting activity in DAergic cells (Murase et al., 1993). Thus, for the groups predicted to display heightened NMDA activation, i.e., increased firing rates and bursting among PFC pyramids (i.e. GDX and GDX-E), it can be further predicted that increased firing rates and elevated bursting activity will also be observed among the DAergic and perhaps non DA neurons of the VTA. Such increases, especially in

bursting activity, would fit with the observation that in GDX and GDX-E rats, extracellular basal PFC DA levels are nearly 2-fold greater compared to control males (Aubele and Kritzer, 2011), as increases in bursting among DA neurons in particular is highly efficient and effective in increasing DA efflux (Grace, 1991; Garris et al., 1993; Floresco et al., 2003).

To test these electrophysiological predictions of the model and to further define the mechanisms of hormone regulation of PFC DAergic innervation, single units were extracellularly recorded from 1) VTA-projecting PFC pyramidal cells confirmed via the collision test and 2), VTA DAergic and fast-spiking non DA neurons identified by conservative waveform criteria (Grace and Bunney, 1984) to compare firing properties in urethane-anesthetized adult male, female, GDX, GDX-TP, and GDX-E rats. In addition, in order to explore the roles of NMDAR-mediated signaling in shaping these group-specific firing phenotypes, recordings of DAergic VTA cells were made before and after intraPFC infusion of the NMDAR selective antagonist APV. Finally, as a control, activity in DA neurons of the substantia nigra was also evaluated in each group. Unlike the DA cells in the VTA, the DA neurons of the SN do not receive cortical innervation from AR-enriched pyramidal cell populations (Kritzer, 1997). Likewise, GDX is without measureable effect on axon innervation density in striatum or motor cortices and has no impact on DA overflow in primary motor cortex (Kritzer et al., 1999; Kritzer, 2000; Aubele and Kritzer, 2011). In sum, these cells show none of the same evidence for susceptibility to hormone/androgen regulation. Therefore, SN DAergic cell firing rates are expected to be similar in males and females and unaffected by GDX.

Materials and Methods

Animal Subjects

A total of 14 adult male, 12 GDX male, 5 GDX-TP male, 7 GDX-E male, and 9 female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Animals were housed in a specific pathogen-free environment in same sex/treatment pairs under a 12/12 h light/dark cycle (lights on at 0700) with food (Purina PMI Lab Diet: Prolab RMH 3000) and water available *ad libitum*. Animals weighed between 300 and 375 grams at the time of electrophysiology. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize animal use and discomfort.

Surgeries

Twenty-eight days prior to electrophysiological testing, male rats underwent GDX or sham surgery. Both surgical procedures were performed under aseptic conditions using intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. For sham and GDX surgeries, an incision was made into the scrotum. For GDX, the vas deferens was bilaterally ligated and both testes were removed. For hormone-supplemented animals slow-release pellets containing either testosterone propionate or 17β-estradiol (Innovative Research of America, Sarasota, FL) were implanted within the tunica. All incisions were closed with wound clips, which were removed after 10 days. Rats were given subcutaneous injections of buprenorphine (0.03 mg/kg) post operatively before being returned to home cages.

On the day of the experiment, the subjects were anesthetized with urethane (1.3 g/kg) injected intraperitonealy. Once fully anesthetized, rats were placed in a stereotaxic apparatus and burr holes were drilled over the VTA, SN, and PFC. Following surgery, core body temperature was maintained by a heating pad.

Hormone Replacement and Estrous Cycle Determination

Male rats were implanted with slow release pellets at the time of GDX. The testosterone propionate (TP) pellets used released 3-4 ng of TP per milliliter of blood per day and the 17β-estradiol (E) pellets used released 25 pg of E per milliliter of blood per day; both have been used previously in this and other labs and have been shown to produce sustained plasma hormone levels falling within physiological ranges (Collins et al., 1992; Adler et al., 1999; Kritzer, 2000). The efficacies of GDX and hormone replacement were verified in quantitative analyses of the weights of animals' androgen-sensitive bulbospongiosus muscles (BSMs) (WAINMAN and SHIPOUNOFF, 1941). The estrous cycle stage of female rats was assessed each day following electrophysiological recording via vaginal lavage and vaginal cytology (Marcondes et al., 2002; Goldman et al., 2007).

Electrophysiological Recordings

Glass electrodes were pulled from 1.2 mm o.d./0.68 mm i.d. glass tubing (A-M Systems, Sequim, WA) using a vertical electrode puller (Narishige, East Meadow, NY) and filled with 2 M NaCl. Electrode tips were then broken back until impedance was

between 4-8 MΩ. Tungsten electrode (A-M Systems) impedance was 0.5 MΩ. Glass electrode signals were amplified by a head stage (Axon Instruments, Sunnyvale, CA) connected to a multipurpose microelectrode amplifier (gain x 1,000) (Axon instruments). Tungsten electrodes were connected to an AC differential amplifier (A-M Systems)(gain x 1,000). For both amplifiers, high- and low-pass filters were set to 0.1 and 10 kHz, respectively. Recordings were monitored by an oscilloscope (Tektronix, Beaverton, OR) and audio monitor (Grass, Warwick, RI) and were digitized with IGOR Pro software (Wavemetrics, Portland, OR).

For VTA and SN recordings, glass electrodes were lowered from the brain surface (coordinates VTA: 5.3 mm posterior to bregma, 0.3-0.8 mm lateral to midline; SN: 5.3 mm posterior to bregma, 1.0-2.5 mm lateral to midline) into the respective regions (depth VTA: 6.5 mm; SN: 6.0 mm) using a hydraulic microdrive (David Kopf Instruments, Tujunga, CA). Action potentials from DA cells were recorded from 6.5-8.5 mm for the VTA and 6.0-8.5 for the SN (with depths depending on laterality for SN recordings). Afterwards, the electrode was retracted and moved 300 μm along the mediolateral or antero-posterior axis to a new location.

For PFC recordings, glass electrodes were lowered from the brain surface (coordinates: 3.2 mm anterior to bregma, 0.2 mm lateral to midline) to a depth of 3.5 mm and recordings were taken from 3.5-5.5 mm. To determine whether the PFC neurons were projecting to the VTA, a tungsten electrode was lowered into the VTA (coordinates same as above) and 15-25 mA stimulation was delivered through a stimulator (World Precision Instruments, Sarasota, FL) at 1 Hz. Only data from PFC

cells that were driven by the VTA stimulation and confirmed via collision test were analyzed (Fig 2.1).

For drug effect studies, a chemotrode (Plastics One, Roanoke, VA) was lowered into the medial PFC and a glass electrode was lowered into the VTA as per the specifications described above. Once a VTA DA cell was located and a stable baseline firing rate was established, APV (10 μg/μL) was administered through the chemotrode at a rate of 0.5 μL/min for 1 min while the DA cell was monitored. Mean firing frequencies were binned per minute following drug administration.

Euthanasia and Histology

All rats were euthanized by rapid decapitation. Brains were removed and post-fixed for 2-4 days in a 10% buffered formaldehyde solution containing 30% sucrose for cryoprotection. Once fixed, brains were frozen in powdered dry ice and serially sectioned in a coronal plane on a freezing microtome (40 μm). A 1 of 4 series of sections were slide mounted and counter-stained with 0.5% cresyl violet. Light microscopic evaluation was used to map electrode tracks. Only those cases where electrodes were confirmed to have hit the VTA, SN, or the deep layers of the left prelimbic and infralimbic medial PFC were included in the analysis.

Data Analysis

The following measures were analyzed offline using IGOR Pro software (Wavemetrics): the number of spontaneously active cells (average number of cells

recorded per electrode track; VTA DA cells only), the firing rate (average number of spikes/second per cell), the percentage of spikes fired within bursts (defined below) per cell, the number of spikes per burst, the average firing rate for spikes not involved in bursts (VTA DA cells only), and the inter-burst interval (seconds between bursts, VTA DA cells only). For VTA and SN recordings DA cells were determined by criteria defined by Ungless and Grace (2012) with action potential widths measuring 2-3ms. Bursts were defined by the criteria of Grace and Bunney (1984) where an interspike interval (ISI) of 12.5 Hz marked the start of a burst and an ISI of 6.25 Hz marked the end of a burst. Pyramidal cells in the PFC were determined through action potential width (≥ 1.0ms) and bursts were defined as beginning once an instantaneous frequency of 40 Hz was reached and ended when the instantaneous frequency fell below 20 Hz (Chang et al., 2010). Group comparisons of the 4 metrics listed above were made with one-way analyses of variance (ANOVA) and Bonferroni post-hoc analyses when allowed. For the drug study, statistics were not performed as there was only one subject for the GDX group.

Results

Effectiveness of Hormone Treatments in Males

The weights of the androgen sensitive bulbospongiosus muscles (BSM) in the male rats showed group differences that paralleled expected differences in circulating androgen levels. Thus, muscle weights in the CTRL and GDX-TP groups were on average 1.7g and 1.8g, respectively, while in GDX and GDX-E rats average muscle

weights were 0.53g and 0.55g, respectively (Fig 2.2). Statistical comparisons of individual rats' muscle weights (one-way ANOVA) identified significant main effects of group [$F_{3,29}$ = 75.63, p < 0.001] on muscle mass and allowed post hoc comparisons confirmed that BSM weights of CTRL and GDX-TP rats were similar to each other; that the BSM weights of GDX and GDX-E rats were similar to each other; and that mean muscle weights of the CTRL and GDX-TP rats were significantly larger than those of both the GDX and GDX-E groups (p < 0.001, see Fig 2.2).

Sex and Hormone Effects on Activity and Firing Patterns of VTA DA Cells

Representative traces of DAergic VTA cell recordings are shown in Figure 2.3 along with average waveforms. In CTRLs (12 cells), roughly 1.5 ± 0.4 spontaneously active DA cells were observed per VTA track (Fig 2.4A) and the mean firing rate was 4.9 ± 0.6 Hz (Fig 2.4B). Approximately 10% of the spikes were involved in bursting activity (Fig 2.4C) with the average number of spikes per burst being 2.5 ± 1.5 (Fig 2.4D). The mean firing rate of spikes not involved in bursting activity was 3.8 ± 0.6 Hz (Fig 2.4E) and the average inter-burst interval was 30 sec (Fig 2.4F). Female rats (3 cells) displayed similar firing patterns with the number of spontaneously active DA cells averaging at 1.0 ± 0 and mean firing rates averaging 4.8 ± 0.6 Hz (Fig 2.4A,B). Bursting activity, however, was different between the sexes as VTA DA cells of female rats were found to burst approximately 3% of the time and had 4.8 ± 0.08 spikes per burst (Fig 2.4C,D). The mean firing rate of non-bursting spikes was faster than that of CTRLs at an average of 5.1 ± 0.7 Hz and the inter-burst interval was comparatively shorter at an average of 21 sec. Conversely, DA cells in the VTA of GDX rats (20 cells) showed

significantly higher levels of activity across all parameters. Thus, the number of spontaneously active cells per track was greater (2.7 ± 0.3), the average firing rate was higher (8.8 ± 0.8Hz), and bursting activity was greater as observed in both percent of spikes involved in bursts (~25%) and spikes per burst (5.0 ± 0.95) (Fig 2.4). The frequency of spikes not involved in bursting activity was also higher than that of CTRLs at an average of 5.0 ± 1.2 Hz and the inter-burst interval was considerably shorter at an average of 5 sec (Fig 2.4). This elevated activity of VTA DA cells was rescued by TP as GDX-TP rats (7 cells) showed numbers of spontaneously active cells (1.5 ± 0.3), mean firing rates (4.5 ± 1.0Hz), percent bursting (1.1%), spikes per burst (3.1± 0.1), non-bursting freq (3.8 ± 0.6 Hz), and inter-burst interval (27 sec) that were similar to or lower than CTRLs (Fig 2.4). However, a rescuing effect of E was not found. Across all measures, VTA DA cells in GDX-E rats (8 cells) displayed firing patterns similar to those of GDX rats. Thus, the number of spontaneously active cells (2.4 ± 0.4), mean firing rates (9.2± 2.1Hz), percent bursting (17%), spikes per burst (3.8± 0.3), and non-burst frequency (5.2 ± 0.4 Hz) were indistinguishable from GDX rats (Fig 2.4). The inter-burst interval (22 sec), however, was more similar to CTRLs than GDX rats. One-way ANOVAs found significant main effects of sex/treatment for the mean firing rates ($F_{4,47}$=4.015, p = 0.007), and inter-burst interval ($F_{4,18}$=4.396, p = 0.012), near significant differences for number of spontaneously active cells ($F_{4,54}$=2.340, p = 0.068) and percent bursting ($F_{4,47}$=2.232, p = 0.081), but no significant difference for spikes per burst or frequency of spikes not involved in bursting activity. Allowed post hoc comparisons showed that for mean frequency, DA cells in GDX and GDX-E rats

displayed higher activity levels compared to those of CTRLs (p = 0.004-.010) and for inter-burst interval GDX rats had shorter intervals compared to CTRL rats (p = 0.006).

Sex and Hormone Effects on Activity and Firing Patterns of SN DA Cells

Unlike DA cells in the VTA, SN DA cells displayed similar firing patterns across all groups. Thus, the mean firing rates ranged from 14.0-19.3 Hz, percent bursting from 48-51%, and spikes per burst from 4.3-5.3 (CTRL: 6 cells, GDX: 4 cells, FEM: 12 cells; Fig 2.5). No significant main effects of sex/treatment were found.

Sex and Hormone Effects on Activity and Firing Patterns of PFC Pyramidal Cells

Representative traces of VTA-projecting PFC pyramidal cell recordings are shown in Figure 2.6. In CTRL rats (9 cells), PFC pyramidal cells had average firing rates of 6.2 ± 1.0 Hz, 0.35% ± 0.15 of spikes involved in bursts, and 3.7 ± 0.3 spikes per burst (Fig 2.7). Pyramidal cells of female rats (2 cells) displayed similar mean firing rates (8.3 ± 3.3 Hz), higher percent bursting (5.92% ± 5.92), and 3 average spikes per burst (Fig 2.7). Conversely, GDX rats (19 cells) were found to have pyramidal cells with significantly elevated levels of activity. Thus, compared to CTRL rats, the GDX group had a greater mean frequency (13.3 ± 2.3 Hz), a greater percentage of spikes involved in bursting activity (6.2% ± 3.9), and a greater number of spikes per burst (4.0 ± 0.8) (Fig 2.7). For all measures, TP (6 cells) was shown to rescue the effects of GDX as measures of mean frequency (8.4 ± 4.2), percent bursting (3.8% ± 3.3), and spikes per burst (3.7 ± 0.1) were all found to be similar to CTRL rats (Fig 2.7). However, the activity levels of PFC pyramidal cells of GDX-E rats (11 cells) were found to be elevated

compared to CTRLs as observed in measures of mean firing rates (15.0 ± 2.7), percent bursting (1.6% ± 0.9), and spikes per burst (3.2 ± 0.09) (Fig 2.7). One-way ANOVAs revealed significant main effects of sex/treatment for mean firing rates ($F_{4,97}$=4.480, p = 0.002) and spikes per burst ($F_{4,44}$=2.840, p = 0.037), but not for percent bursting. Allowed post hoc comparisons found that for measures of mean frequency, GDX and GDX-E rats had significantly elevated levels of activity compared to CTRL rats ($p \leq$ 0.001-0.003), while for measures of spikes per burst, GDX and GDX-E rats averaged slightly fewer compared to CTRLs (p = 0.004-0.006).

Hormone Effects on NMDA Receptor Regulated Firing Patterns of VTA DA Cells

Following administration of APV in the PFC of CTRL male rats, the mean frequency of VTA DA cells was found to increase by 40% within the first minute of drug administration and remained above baseline levels for 16 minutes (Fig 2.8). Conversely, in the GDX rat, the mean firing rate of VTA DA cells decreased by 80% following drug administration and this depression in activity remained below baseline for over 20 minutes (Fig 2.8).

Discussion

Sex differences in DA-dependent PFC-mediated operations are known to exist in both healthy function and their dysfunction in disease. Previously, we have shown that both sex and sex hormones affect the NMDAR-mediated regulation of PFC DA levels such that in male and GDX-TP rats, NMDA has an inhibitory influence on PFC DA

levels and in groups with low circulating androgen levels (i.e. female, GDX, and GDX-E rats) NMDA has an excitatory influence (Aubele and Kritzer, 2012). A working model of the mechanisms involved in PFC DA regulation proposes that androgen suppresses the NMDAR-mediated influence on VTA-projecting PFC pyramidal cells, which have been found to have high concentrations of AR (Aubele and Kritzer, 2012). Thus, low circulating androgen levels lead to heightened NMDA-mediated excitability of the PFC pyramids that drive DAergic cells in the VTA. Here, we directly observed the firing patterns of VTA-projecting PFC pyramidal cells and DAergic cells in the VTA of CTRL, female, GDX, GDX-TP, and GDX-E rats. For comparison, DAergic cells in the SN, which receive cortical inputs with much less AR enrichment comparatively, were also recorded. Finally, the effect of NMDA antagonists on the firing rates of VTA DA cells in CTRL and GDX rats was also tested.

In this study, both VTA-projecting PFC pyramidal cells and VTA DAergic cells were found to have similar firing patterns in males and females while GDX rats displayed elevated activity across all parameters. In addition, TP, but not E, was found to rescue GDX effects such that cells recorded from GDX-TP rats displayed firing rates similar to those of CTRL and those of GDX-E rats had firing rates similar to those of GDX rats. Further, as expected, SN DAergic cells showed similar firing patterns across all groups. Finally, following infusion of APV into the PFC, VTA DAergic cells of control males showed an increase in firing rates and bursting activity while GDX rats showed a decrease in both. Below, these findings will be discussed in relation to the intra-PFC mechanisms regulating this circuitry as well as the effects of tonic and phasic DAergic innervation of the PFC on PFC function.

Sex Hormone Effects on PFC Function

The findings presented here show GDX-induced increases in the mean firing rates and bursting activity of both PFC pyramidal cells and VTA DAergic cells, an effect further found to be androgen sensitive and estrogen insensitive. This elevated activity of the circuitry regulating PFC DA tone helps elucidate previous findings that GDX induces significant increases in basal PFC DA levels in a similarly androgen sensitive, estrogen insensitive manner (Aubele and Kritzer, 2011). These increases in activity and basal DA level likely contribute to the profound deficits observed in the DA-dependent PFC–mediated functions of GDX rats. A significant body of work has explored the effects of GDX on the executive functions of the PFC and has found that GDX induces deficits in spatial working memory (Gibbs and Johnson, 2008; Spritzer et al., 2008), novel object recognition (Aubele et al., 2008), and perseveration (Kritzer et al., 2007). Less work has been done to test whether these effects are androgen or estrogen dependent, but the studies that have explored this have largely found these effects to be primarily androgen sensitive (Kritzer et al., 2007; Aubele et al., 2008; Spritzer et al., 2011). These functions have been shown to rely heavily on PFC DA levels, which must be kept within a narrow window in order to maintain optimal PFC function (Murphy et al., 1996; Zahrt et al., 1997; Morrow et al., 2000; Moghaddam and Jackson, 2004; Winter et al., 2009) and the accelerated firing rates of DAergic neurons in GDX and GDX-E rats perturbs this DA balance. Under normal conditions, the PFC-projecting DAergic cells of the VTA fire single spikes (Grace and Onn, 1989; Overton and Clark, 1997). However, when salient stimuli are presented, these cells show elevated bursting activity, resulting

in phasic increases in PFC DA tone (Freeman and Bunney, 1987; Overton and Clark, 1997; Hyland et al., 2002). Thus, in GDX and GDX-E rats, whose DAergic cells display significantly greater basal firing rates and greater bursting activity, it is possible that the signal for salience is lost, thereby disrupting performance in behavioral tasks testing PFC function.

Tonic Dopamine Innervation and the Prefrontal Cortex

As functions mediated by the PFC depend on both tonic DAergic innervation as well as its phasic changes, alterations in the tonically maintained basal DA levels could alter responses to the phasic fluctuations as well. In fact, long-term changes in tonic DAergic innervation have been shown to result in alterations in homeostatic mechanisms such as DA receptor expression (Creese et al., 1977; Creese and Snyder, 1979; Mishra et al., 1980; MacKenzie and Zigmond, 1984, 1985), autoreceptor-mediated regulation of DA synthesis (Farnebo and Hamberger, 1971; Hefti et al., 1980; Altar et al., 1987; Stachowiak et al., 1987), as well as changes in DAergic axon sprouting (Onn et al., 1986). These alterations in DA signaling shift the signal to noise ratio between tonic and phasic DA levels and can cause aberrant neurochemical and physiological responses to phasic DA level changes (Grace, 1991). One would also expect these changes to also alter PFC-mediated behaviors dependent on DA homeostasis. Thus, the androgen sensitive elevations in the firing rates and bursting behavior of the PFC-VTA-PFC circuitry reported here are likely to not only affect the tonic DA innervation of the PFC and basal PFC DA levels, but also the PFC response to phasic changes in this innervation.

Sex Effects on PFC Function

In Chapter I, it was shown that unlike male rats, female rats show decreases in PFC DA levels following NMDA receptor antagonism in the PFC. Thus, a working model was proposed wherein, similar to GDX rats, low circulating androgen levels in female rats lead to a comparatively greater NMDA-mediated influence on VTA projecting pyramidal cells. However, it was also shown in Chapter I that basal PFC DA levels were similar between male and female rats, likely a result of the greater GABA-B mediated inhibition observed in the PFC of female rats. As predicted based on these findings, firing rates and bursting activity of both VTA-projecting pyramidal cells and DAergic VTA cells were similar across sex.

Though no differences were observed in DA levels or firing patterns, sex differences have been established in PFC-mediated behaviors. For example, female rats show superior performances on tests of impulsivity while males outperform females on attentional tasks (Jentsch and Taylor, 2003; Bayless et al., 2012). Further, though estrous cycle stage was not found to have an effect on firing patterns in this study, others have found performances on working memory tasks to fluctuate across the estrous cycle (Warren and Juraska, 1997; Healy et al., 1999; Pompili et al., 2010). Thus, though estrogen was not found to have an effect here, and E was not found to have an effect in the GDX male rats, it is possible that estrogenic effects are taking place outside of the cells recorded here. Evidence for this is found in studies that have shown E to have a rescuing effect on GDX-induced behavioral deficits in male rats (McConnell et al., 2012). Therefore, additional studies need to be done in order to

elucidate the sex differences observed in PFC-dependent behaviors as well as the potential roles estrogen plays in modulating them. A potential substrate could be the GABAergic interneurons within the PFC. In Chapter I, it was shown that both females and GDX-E rats display heightened GABA-B mediated inhibition in the PFC compared to male rats. Thus, physiological studies of sex and gonadal hormone effects should be expanded to the inhibitory interneurons of the PFC.

The sex and sex hormone effects on PFC pyramidal cell and DAergic VTA cell physiology shown here and the effects on the GLUergic and GABAergic PFC DA regulatory mechanisms discussed in Chapter I will likely affect PFC-dependent behaviors. To test this, spatial working memory, a PFC-mediated function, was tested on the Barnes maze in the third and final chapter of this dissertation.

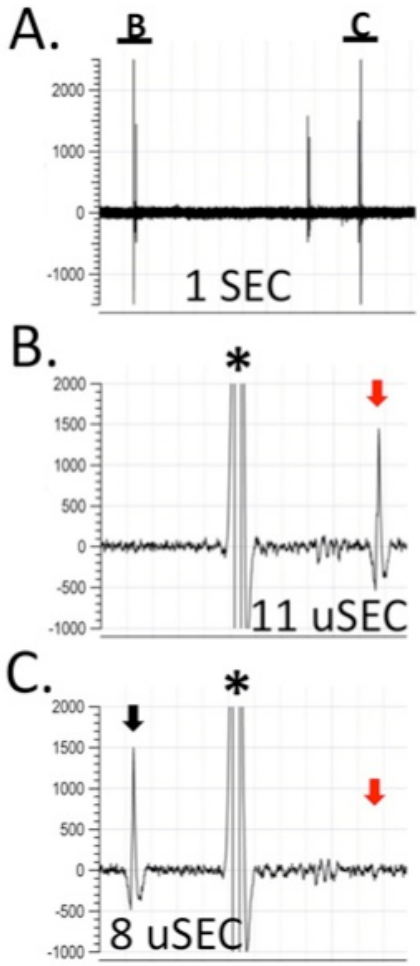

Figure 2.1. Representative single unit recording from VTA-projecting PFC pyramidal cell demonstrating the collision test. Collision testing: following stimulation of the VTA, PFC unit is identified by antidromic stimulation (B, red arrow) and collision extinction (C, red arrow) of spontaneously occurring spikes (black arrow). Asterisks mark stimulation artifacts.

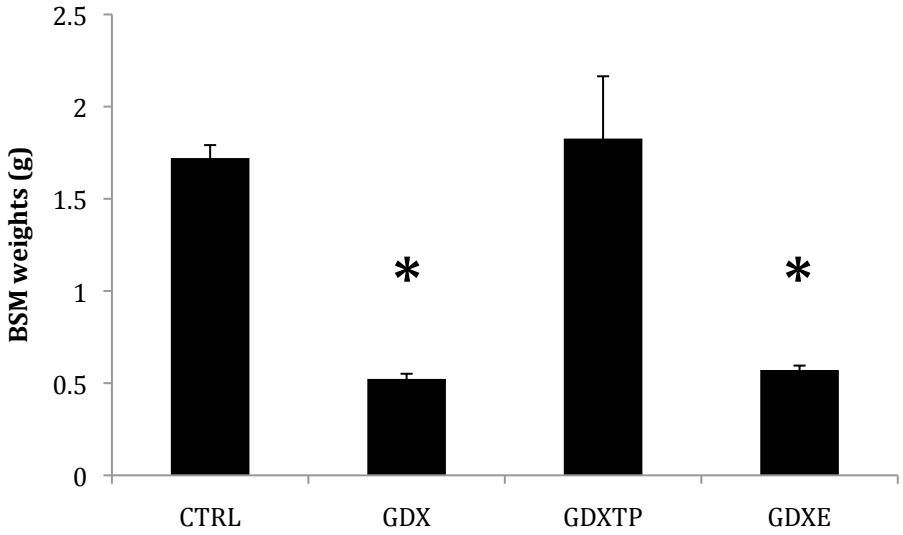

Figure 2.2. Bar graphs showing average bulbospongiosus muscle weights in grams (g) for gonadally intact control (CTRL), gonadectomized (GDX), and gonadectomized male rats supplemented with testosterone propionate (GDX-TP) or estradiol (GDX-E). Muscle weights of CTRL and GDX-TP rats were similar to each other and were significantly greater than those of GDX and GDX-E rats. Muscle weights of GDX and GDX-E rats were also similar to each other. Error bars represent standard errors of the mean. Asterisks denote significant differences from CTRL for post-hoc testing at the $p < 0.05$ level

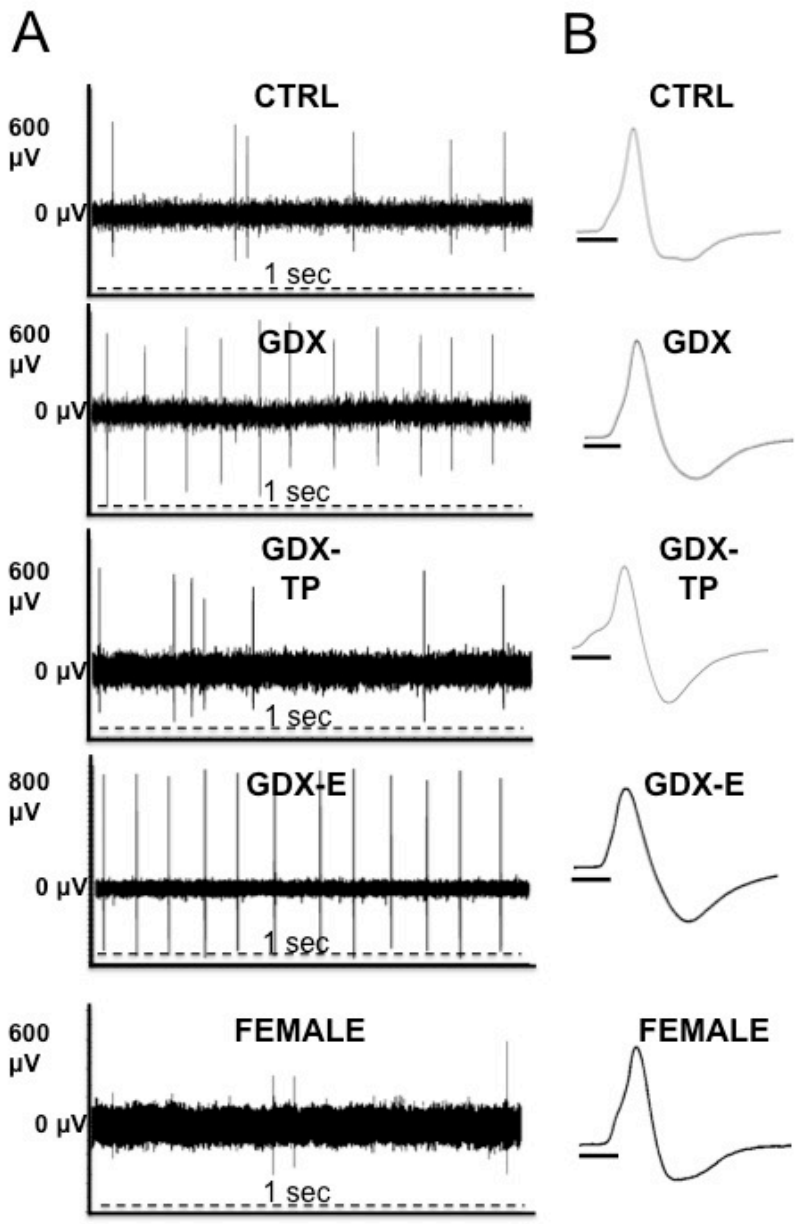

Figure 2.3. Representative traces (A) and waveforms (B) of spontaneously active dopaminergic cells in the ventral tegmental area of CTRL, GDX, GDX-TP, GDX-E, and FEMALE rats. (A) Representative traces are 1 second in length. (B) Scale bars are 1 ms in length.

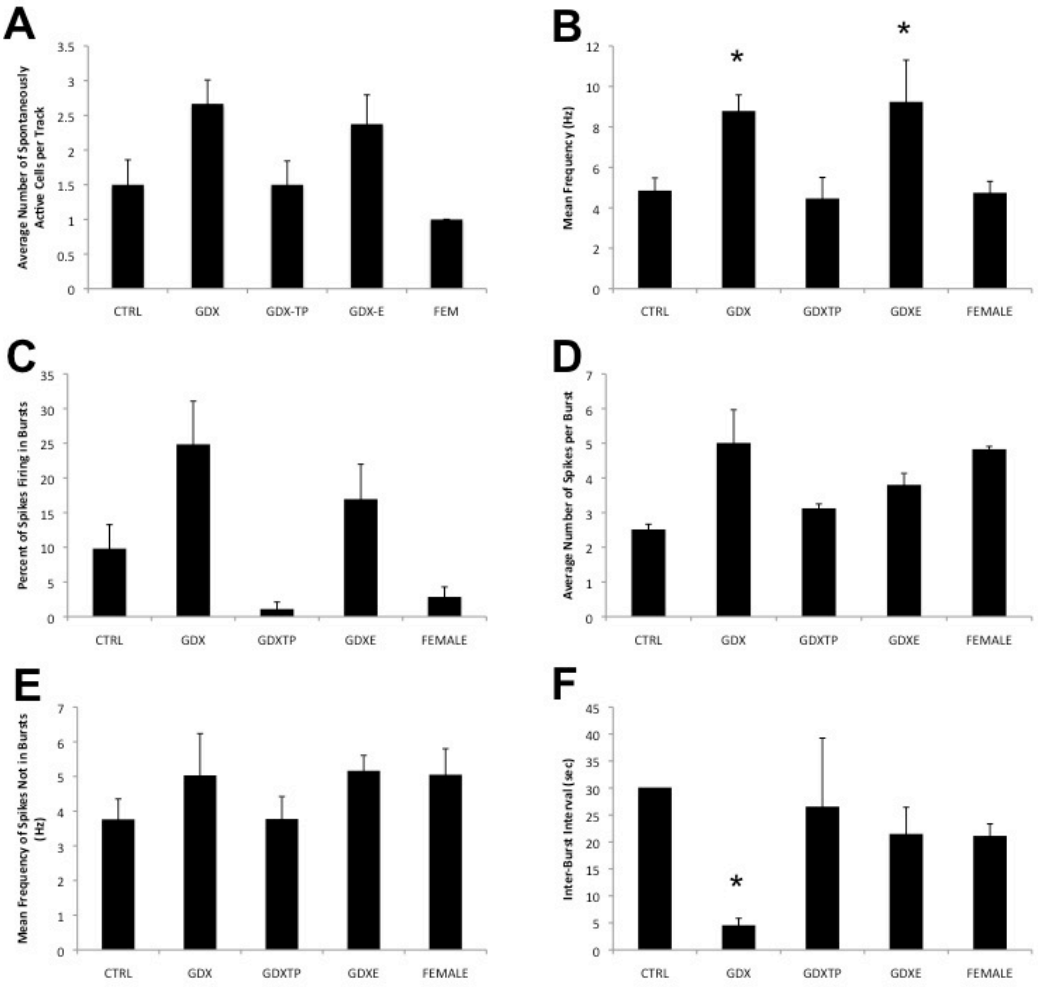

Figure 2.4. Bar graphs showing (A) the average number of spontaneously active DAergic VTA cells per recording track, (B) the mean frequencies, (C) the percentage of spikes firing in bursts, (D) the average number of spikes per burst, (E) the mean frequency of spikes not firing in bursts, and (F) the inter-burst interval. Group differences approached significance in the number of spontaneously active cells and bursting activity. Group differences were found in mean frequency and inter-burst interval and on average, cells in GDX and GDX-E rats firing more rapidly than those of CTRL rats while GDX rats had significantly shorter inter-burst intervals. Error bars represent standard errors of the mean. Asterisks denote significant differences from CTRL for post-hoc testing at the $p < 0.05$ level.

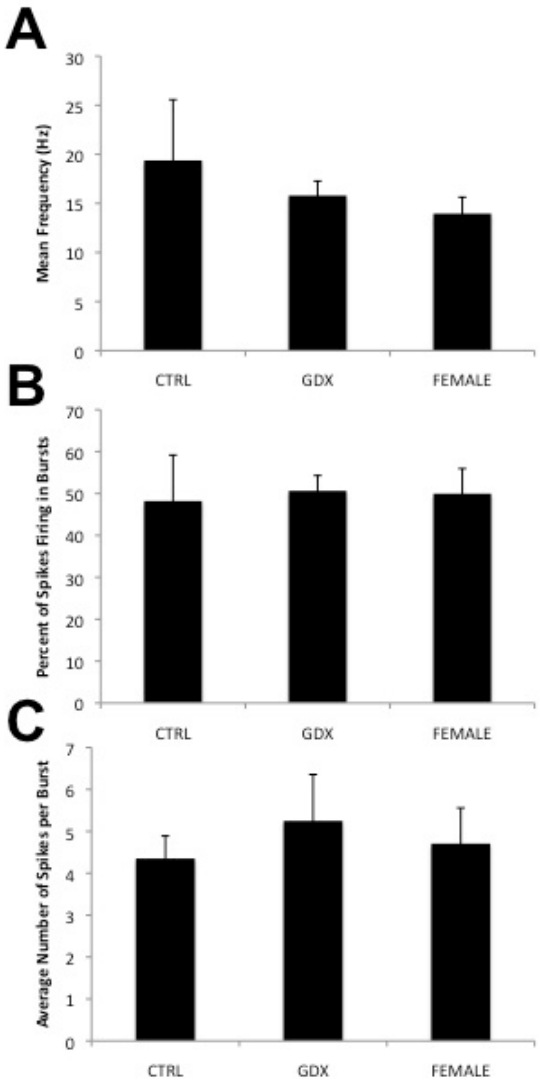

Figure 2.5. Bar graphs depicting the (A) mean frequencies of DAergic substantia nigra cells, (B) the percent of spikes firing in bursts, and (C) the average number of spikes per burst. No differences were found on any measure between CTRL, GDX, and FEMALE rats. Error bars represent standard errors of the mean.

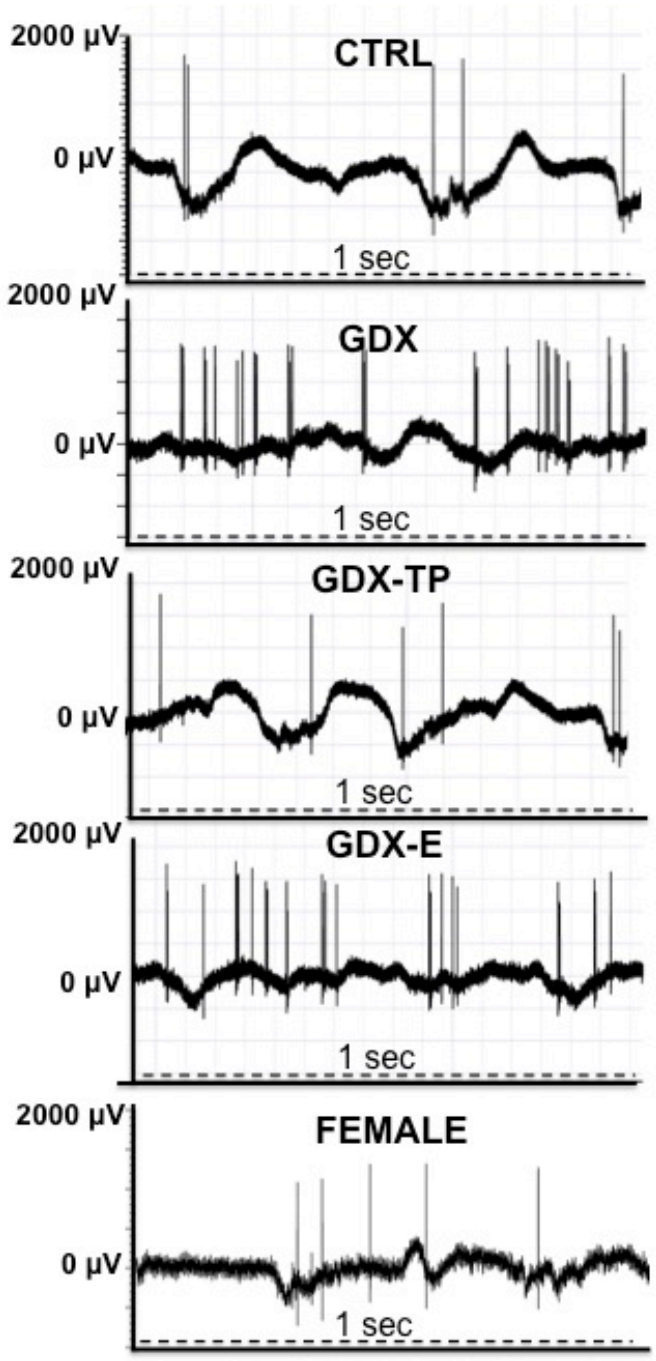

Fig 2.6. Representative traces of spontaneously active prefrontal cortical pyramidal cells recorded from CTRL, GDX, GDX-TP, GDX-E, and FEMALE rats. Traces are 1 second in length.

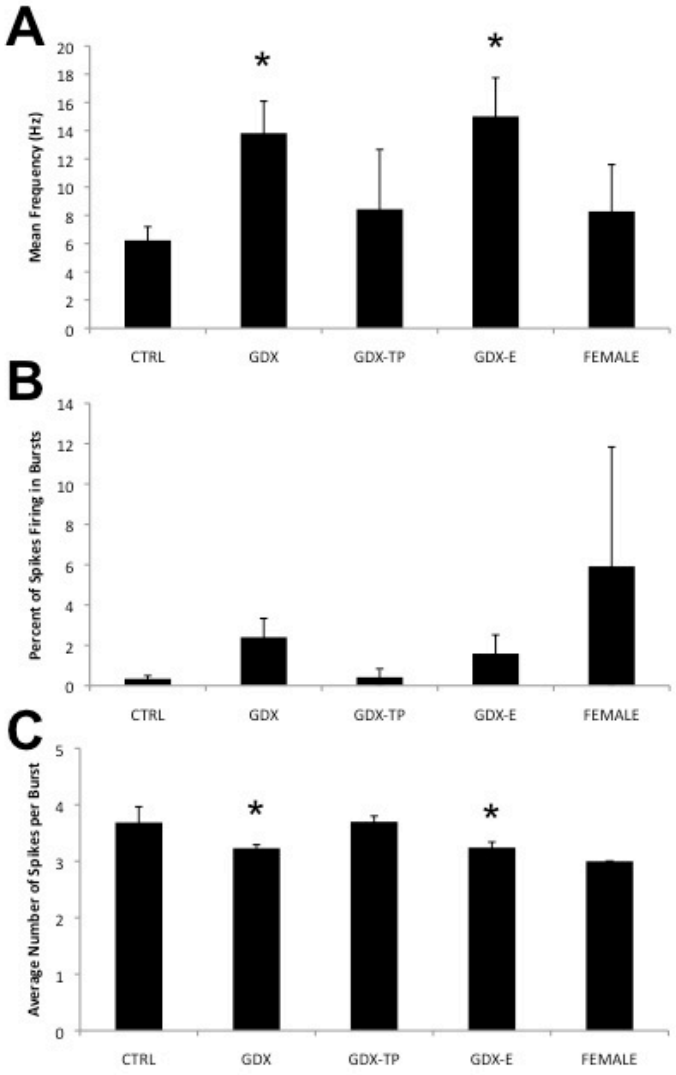

Figure 2.7. Bar graphs depicting the (A) mean frequencies of VTA-projecting pyramidal cells, (B) percentage of spikes fired in burst, and (C) the average number of spikes per burst. GDX and GDX-E rats fired at faster frequencies compared to CTRL but had fewer spikes per burst. Error bars represent standard errors of the mean. Asterisks denote significant differences from CTRL for post-hoc testing at the $p < 0.05$ level.

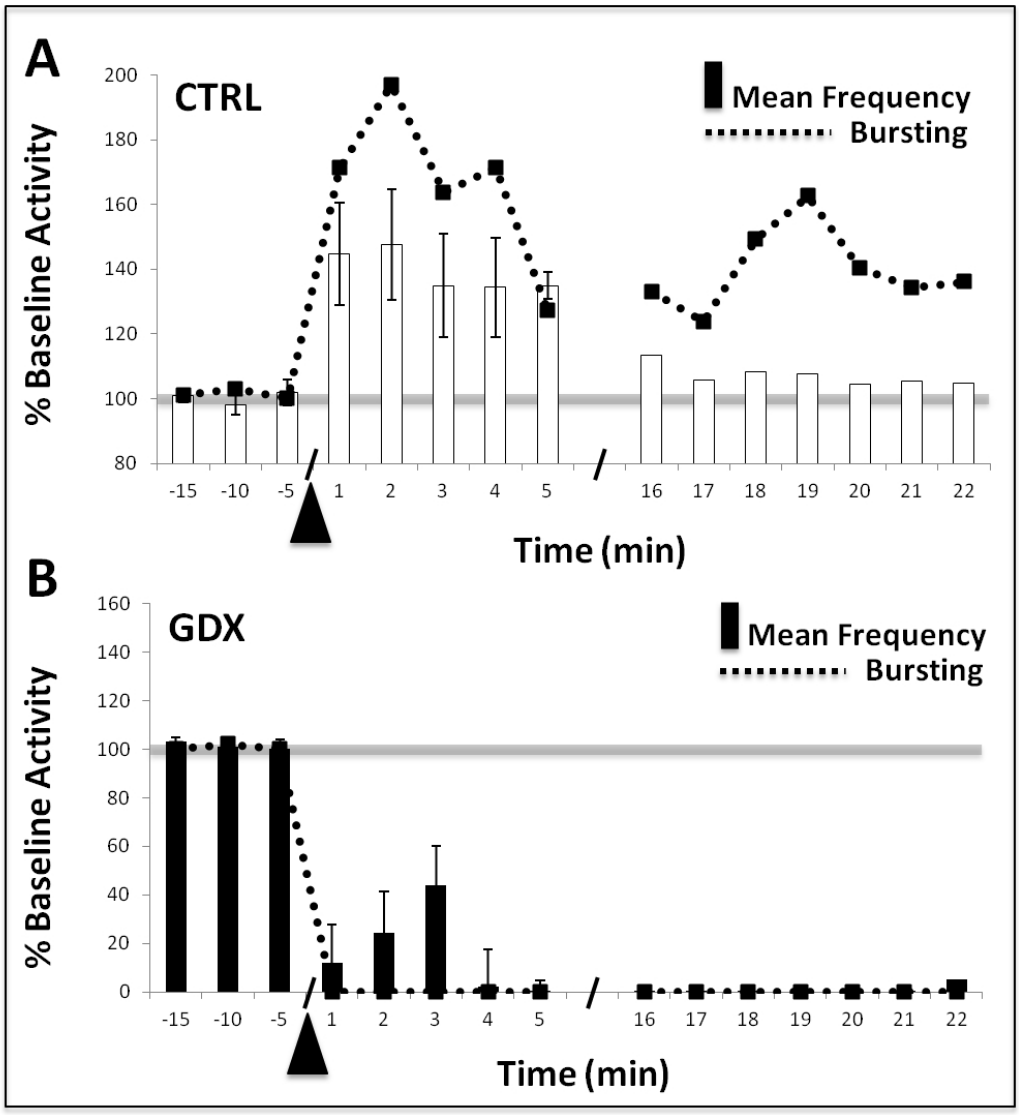

Figure 2.8. Bar and line graphs showing average changes in firing frequency and bursting activity per minute of DAergic VTA cells before and after intraPFC infusion of the NMDA antagonist APV. In CTRL rats, APV resulted in increases in both average firing rates (bars) and bursting activity (dotted line), while in GDX rats both of these measures were suppressed by APV. Black triangles denote when APV was infused. Error bars represent standard errors of the mean.

Chapter III

Assessment of spatial cognition in adult male, adult female, and adult male rats that were gonadectomized, or gonadectomized and supplemented with 17β-estradiol or testosterone propionate using the Barnes maze

As described previously, in humans, sex differences have been identified for a number of cognitive functions (Mann et al., 1990; Weiss et al., 2003; Lejbak et al., 2011; Herlitz et al., 2013; Talarowska et al., 2013). Sex differences have also been documented in the cognitive dysfunction that characterizes numerous neurological and psychiatric disorders (Leung and Chue, 2000; Miller and Cronin-Golomb, 2010; Vaskinn et al., 2011; Han et al., 2012). Particularly robust are the consensus findings for a male advantage in spatial tasks that range from mental object rotation (Hampson, 1990b; Moffat and Hampson, 1996; Parsons et al., 2004; Kaufman, 2007) to virtual Morris water maze and radial arm maze tasks (Astur et al., 1998; Moffat et al., 1998; Astur et al., 2004; Coluccia and Louse, 2004; Woolley et al., 2010). Further, positive correlations have been identified between measures of spatial ability and circulating testosterone levels in men and women (Gordon and Lee, 1986a; Christiansen and Knussmann, 1987; Janowsky et al., 1994; Silverman et al., 1999; Duff and Hampson, 2000), while negative correlations have been reported between estrogen levels and spatial cognition in women across the menstrual cycle (Hausmann et al., 2000; Simic and Santini, 2012).

While findings from human studies suggest that both organizational and activational hormone actions influence spatial ability, the exact natures of these actions have yet to be fully resolved. However, the numerous studies in animal and especially rodent models that have sought to clarify these issues have yet to reach complete consensus. For example, while a recent meta analysis supports a male over female advantage in spatial working and spatial reference memory in rats (Jonasson, 2005), the extant literature also includes studies finding no sex differences (Juraska et al., 1984; Kolb and Cioe, 1996; Healy et al., 1999; Faraji et al., 2010) or more infrequently superior spatial performance in females (Gibbs and Johnson, 2008) on spatial behaviors. Findings related to activational hormone effects from studies of gonadectomized (GDX), ovariectomized, and hormone-supplemented rats span a similar gamut from no effects (Singh et al., 1994; Luine et al., 1998; Ziegler and Gallagher, 2005; Sandstrom et al., 2006; Gibbs and Johnson, 2008; Spritzer et al., 2008; Spritzer et al., 2011), to those that differentially identify spatial constructs as estrogen vs. androgen sensitive (Kritzer et al., 2001; Gibbs, 2005; Sandstrom et al., 2006; Kritzer et al., 2007; Spritzer et al., 2011; McConnell et al., 2012).

Several non-mnemonic factors are known to influence outcome measures in studies of sex and/or sex hormone effects on performance in spatial tasks. These include animals' ages (Kanit et al., 2000; Bimonte-Nelson et al., 2003) and hormone status, including the duration and dose of hormone deprivation and replacement (Goudsmit et al., 1990; Bimonte and Denenberg, 1999; Galea et al., 2001; Daniel et al., 2006; Spritzer et al., 2011; Spritzer et al., 2013). In addition, evidence for a male preference in utilization of hippocampal-dependent place strategies (Blokland et al.,

2006; Hawley et al., 2012) and for high levels of testosterone in males and high levels of estrogen in proestrus females in biasing animals to use place rather than response strategies in solving spatial mazes (Korol et al., 2004; Spritzer et al., 2013) identify animals' approaches as yet another factor likely to influence outcomes, particularly across studies using mazes and testing contingencies where advantage is differentially conferred for place, response or other approaches (Healy et al., 1999; Lund and Lephart, 2001; Gibbs and Johnson, 2008; Faraji et al., 2010; Ruprecht et al., 2013).

More recently, it has been suggested that task-related variables of stress and/or reward can also impact outcomes in studies of sex and sex hormone effects on spatial cognition (McConnell et al., 2012). Both factors are known to differentiate and differentially influence behavior in gonadally intact and castrated male and female rats (Heinsbroek et al., 1987; Beiko et al., 2004; Conrad et al., 2004; Kritzer et al., 2007; Luine, 2007; Osborne et al., 2009; Belviranli et al., 2012). Thus, differences in sensitivity to stress and/or reward contingencies could help explain: the negative impact of GDX in appetitively motivated radial arm maze tasks but not aversely motivated Morris water maze tasks (Spritzer et al., 2008; Spritzer et al., 2011); the enhancement of male over female sex differences in spatial navigation in the dry-land ziggurat compared to Morris water maze (Faraji et al., 2010); and the dampening effects that pre-training has on the expression of male over female sex differences in the Morris water maze (Bucci et al., 1995; Perrot-Sinal et al., 1996; Healy et al., 1999; Lukoyanov et al., 1999; Faraji et al., 2010).

To minimize potentially confounding sex- and sex hormone-sensitive factors of stress and reward, a recent study compared short term spatial memory in extensively

habituated control, GDX, and hormone-replaced male rats using a non-rewarded object in place memory task (McConnell et al., 2012). While findings of GDX-induced spatial working memory deficits were similar to those of previous studies (Kritzer et al., 2001; Sandstrom et al., 2006; Kritzer et al., 2007; Gibbs and Johnson, 2008; Spritzer et al., 2008), their attenuation by estrogen as well as by testosterone and dihydrotestoseterone differs from the estrogen-insensitivity that has been found for GDX-induced spatial working memory deficits in rewarded tasks (Kritzer et al., 2001; Kritzer et al., 2007). This raises new questions about how activational hormone actions may influence rats' performances on spatial cognitive tasks and underscores the need for utilization of relatively stress- and reward-neutral testing conditions. Accordingly, we used the Barnes maze, a spatial memory paradigm where behavior is motivated by rodents' natural agoraphobia to search among holes to locate a recessed goal chamber (Barnes, 1979). While holding several advantages for the study of sex and sex hormone impact on spatial cognition, the Barnes maze has rarely been used for these purposes (Ryan and Vandenbergh, 2006; Berry et al., 2008; Barrett et al., 2009; O'Leary et al., 2011). Here the Barnes maze was used to compare multiple measures of performance related to task acquisition, spatial working memory, spatial reference memory, and spatial learning strategies in adult male, adult female, GDX males and GDX male rats supplemented with testosterone propionate (TP) or 17β estradiol (E). Further, in line with the work presented in Chapters II and III, due to the known effects of GDX on NMDA-mediated regulation of PFC DA levels (Aubele & Kritzer, 2012), the effects of GDX on basal extracellular PFC DA levels (Aubele & Kritzer, 2011), and the GDX-induced changes in firing patterns of DAergic VTA cells (Chapter II), additional

groups of intact male, GDX, GDX-E, and GDX-TP rats were also implanted with bilateral cannulae and given infusions of the NMDA antagonist APV prior to the first testing trial in order to test hormone effects on NMDA influence of DA-dependent PFC-mediated behaviors.

MATERIALS AND METHODS

Animals

A total of 82 male and 8 female Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Of the male rats, 21 were gonadectomized (GDX), 21 were GDX and supplemented with testosterone propionate (GDX-TP), 18 were GDX and supplemented with 17β-estradiol (GDX-E), and 22 received sham surgeries (CTRL) 28 days prior to behavioral testing. To allow similar habituation to housing conditions, female rats were housed in the SBU animal facility for similar lengths of time as the male subjects used in this study prior to the commencement of behavioral testing. All were housed in same sex/same treatment pairs under a 12 h, non-reversed light-dark cycle, in standard cages. Ground corncob bedding (Bed O' Cobs, Anderson) was provided and animals had free access to food (Purina PMI LabDiet: ProLab RMH 3000) and water. All animals were roughly 3 months of age and weighed 275-350g at time of testing. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize animal use and discomfort.

Surgeries

Twenty-eight days prior to maze habituation and behavioral testing, male rats underwent GDX or sham surgery. Both surgical procedures were performed under aseptic conditions using intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. For sham and GDX surgeries, an incision was made into the scrotum. For GDX, the vas deferens was bilaterally ligated (sterile non-absorbable, 6-0 silk sutures) and both testes were removed. For hormone-supplemented animals slow-release pellets containing either testosterone propionate or 17β-estradiol (Innovative Research of America, Sarasota, FL) were implanted within the tunica. All incisions were closed with wound clips, which were removed after 10 days. Rats were given subcutaneous injections of buprenorphine (0.03 mg/kg) post operatively before being returned to home cages.

Stereotaxic Placement of Guide Cannulae

For rats receiving drug infusions, craniotomies were performed 4 days before behavioral testing under aseptic conditions and using intraperitoneal injections of ketamine (0.9mg/kg) and xylazine (0.5 mg/kg) as anesthesia. Stereotaxic coordinates were used to place bilateral guide cannulae (14mm, Plastics One, Roanoke, VA) within the left pregenual medial PFC. Cannulae were secured to the skull with shallow anchor screws and dental cement. After surgeries, animals were given single doses of buprenorphine (0.03 mg/kg) and returned to home cages for recovery.

Hormone Replacement and Estrous Cycle Determination

Male rats were implanted with slow release pellets at the time of GDX. The testosterone propionate (TP) pellets used released 3-4 ng of TP per milliliter of blood per day and the 17β-estradiol (E) pellets used released 25 pg of E per milliliter of blood per day; both have been used previously in this and other labs and have been shown to produce sustained plasma hormone levels falling within physiological ranges (Collins et al., 1992; Adler et al., 1999; Kritzer, 2000). The efficacies of GDX and hormone replacement were verified in quantitative analyses of the weights of animals' androgen-sensitive bulbospongiosus muscles (BSMs) (Wainman and Shipounoff, 1941). The estrous cycle stage of female rats was assessed each day following maze testing via vaginal lavage and vaginal cytology (Marcondes et al., 2002; Goldman et al., 2007). Lavage samples were collected in saline using a fire-polished, sterile glass pipette. The sampled fluid was immediately placed on a slide, a coverslip was gently placed over it and cytology was evaluated using light microscopy, differential interference contrast (DIC) optics and a 20x objective. Estrous smears were identified by the predominance of cornified epithelial cells; proestrus smears were identified by the abundance of nucleated, non-cornified epithelial cells; diestrus smears were identified by the predominance of leukocytes along with cornified (diestrus I) or nucleated epithelial cells (diestrus II). On testing Day 1 four rats were in diestrus and four were in proestrus. Twenty-four hours later on testing Day 2, one rat transitioned from proestrus to estrus, two rats transitioned from diestrus to proestrus, and two rats transitioned from proestrus to diestrus (Table 3.1). Two rats remained in diestrus and one in proestrus from Day 1 to Day2 (Table 3.1). On testing Day 7, one week later, five rats were in diestrus and three were in proestrus (Table 3.1).

Behavioral Testing

Apparatus: The Barnes maze consisted of a white, laminate, circular platform, 122 cm in diameter that was mounted on a rotatable pedestal 78 cm above the ground. Along its perimeter were 12 evenly spaced holes, each 10 cm in diameter that were separated 17 cm apart and were located 1.3 cm from the platform edge. The maze also had one removable black acrylic goal box (23 x 17 x 10.2 cm) that could be fitted under any of these holes. The maze pedestal was wrapped in black draping to make all holes, whether or not they contained the goal box, appear dark from the maze surface to minimize visual cues. A removable opaque open cylinder (20.3 cm in diameter, 17.8 cm high) was also used as a start box to position animals in the center of the maze for the start of each trial (see Procedures).

The maze was located in the center of a 12 ft square, sound-attenuated, evenly illuminated room. Bright light, which is sometimes used to motivate rats to locate the goal box was avoided. One wall of the room had fixed high contrast spatial cues (2 large, 10 inch, black triangles). During testing, the experimenter stood in a fixed position in one corner of the room 1.5 m away from the maze. A video camera (Logitech High Definition Webcam) was suspended 144.8 cm above the center of the maze and a laptop computer that operated the video camera was located next to the investigator. The testing room was located within a larger behavioral suite that included a separate, central room where animals were kept in home cages before and between trials.

Testing procedures: Testing began 28 days after GDX or sham surgery in the males and after a 28 day wait following purchase of the females. All animal handling and testing was done by a single, experienced individual (MNL).

1. *Maze Habituation*. Habituation to the maze was a step-wise process carried out in a single session. On the day before formal testing began, rats were transported in their home cages to the central room of the testing suite and were allowed to acclimate for approximately 1 h. After acclimatization, rats were moved (in their home cages) into the room containing the maze with the goal box placed in a specified position. Rats were removed from home cages, placed directly into the recessed goal box and were allowed to remain there undisturbed for 2 min. Rats were then returned to their home cages for a 15 min waiting period. Next, rats were placed on the maze surface adjacent to the goal hole; if they did not spontaneously enter the goal box, they were gently guided into it and were allowed to remain there undisturbed for 2 min. After being returned to the home cage for 15 min, rats were placed at the maze center within a temporarily constructed, opaque walkway that led directly to the goal hole; if rats did not spontaneously approach and enter the goal box, they were gently guided into it and allowed to remain undisturbed for a final 2 min.

2. *Behavioral Testing*. Behavioral testing began 24 h after maze habituation. The goal box was positioned under the hole located 180 degrees from its site during habituation. After rats were acclimated to the testing suite (1 h), rats were taken in home cages into the testing room. Rats receiving bilateral infusions of APV were gently held with a cloth while infusion cannulae (Plastics One) were inserted into guide cannulae. Infusions of APV (10 μg/μL) or saline were given 15 min prior to the start of the first trial and were

infused at a rate of 0.5 μL/min for one min via a syringe pump and controller (Bioanalytical Systems Inc, West Lafayette, IN). Infusion cannulae were allowed to remain in place for an additional minute after drug infusion and were then replaced with dummy cannulae. Testing began by placing rats in the start box (cylinder) located at the center of the maze. After a 10 s delay, the cylinder was lifted away and rats were given 3 min to explore. Upon entering the goal box, rats were allowed to stay undisturbed for 2 min. If rats failed to find the goal location within the 3 min trial, they were gently guided into it, and allowed to remain for 2 min. Rats were then returned to their home cages for a 15 min inter-trial interval. This testing process was then repeated for 3 additional trials to conclude Testing Day 1. Following the same procedures, though without drug or saline infusion, and using the same location of the goal box, rats received an additional 4 trials 24 h later (Testing Day 2) and except for drug infusion groups, one additional trial one week later (Testing Day 7). All maze and goal box surfaces were cleaned with 10% ethanol between each trial and the maze itself was also rotated between trials (but the goal box remained in the same relative position in space) to minimize olfactory cues.

Analysis: Behavior was scored off-line from archived videotaped trials a by a single observer (MFK) who was blind to animal group. Tapes were scored for the quantitative measures listed and described in Table 3.2. For drug infusion groups, these were the only assessments scored.

Rats' paths were also digitized using the nose as a reference point and sampling videotaped trials every 0.17 s (Tracker 4.62, Open Source Physics). These digitized

paths were exported into ImageJ (open source, NIH) as black and white images. Black pixels were quantified, converted into centimeters and used to calculate the following:

Total path length: Total lengths of rats' paths (in centimeters).

Peripheral maze path length: Lengths of rats' paths within 20cm of the maze edge (in centimeters).

Central maze path length: Lengths of rats' paths within a 40cm radius from the center of the maze (in centimeters).

Rats' search strategies were also categorized according to quantitative criteria and statistically compared using Fisher's Exact Test (see below). For each trial, rats' searches were assigned to one of the following categories:

Direct to Goal: Scored when all investigations involved holes that were within three holes from the goal on Day 1 and fewer than 3 errors were made; or within two holes from the goal on Days 2 and 7 and fewer than 2 errors were made.

Serial Search: Scored when 80% of holes investigated were adjacent or within 1 hole of each other, when animals made no changes in direction and remained in peripheral portions of the maze.

Random Search: Scored when 60% of holes investigated were non-adjacent and when animals made more than 2 changes of direction and/or traverses across central portions of the maze.

Statistics

Bulbospongiosus weights were compared across groups using a one-way analysis of variance (ANOVA). Quantitative Barnes maze data were compared across

groups (per testing day) using two-way ANOVAs with repeated measures designs, where sex and hormone treatments (group) served as the independent factor and trials served as the repeated measure. When significant group, trial, or group by trial interactions were found, post hoc Bonferroni analyses were used to identify groups that over or underperformed (collapsing across trial), changes in trial-to-trial performance indicative of learning (collapsing across groups) or trials in which groups' performances diverged, respectively. Paired t-tests were also used in within groups comparisons of outcome measures between i) last trial of Day 1 and the first trial of Day 2 and ii) the last trial of Day 2 and the single Day 7 trial. Fisher's exact tests were used to compare groups in terms of categorical variables of search strategy. In all cases $p < 0.05$ was accepted as significant and $0.05 < p < 0.10$ was defined as near significant.

RESULTS

Effectiveness of Hormone Treatments in Males

The weights of the androgen sensitive bulbospongiosus muscles (BSM) in the male rats showed group differences that paralleled expected differences in circulating androgen levels. Thus, muscle weights in the CTRL and GDX-TP groups were on average 1.8g and 1.6g, respectively, while in GDX and GDX-E rats, average muscle weights were 0.3g and 0.5g, respectively (Fig 3.1). Statistical comparisons of individual rats' muscle weights (one-way ANOVA) identified significant main effects of group [$F_{(3,24)} = 19.73$, $p < 0.001$] on muscle mass and allowed post hoc comparisons confirmed that BSM weights of CTRL and GDX-TP rats were similar to each other; that the BSM weights of GDX and GDX-E rats were similar to each other; and that mean muscle

weights of the CTRL and GDX-TP rats were significantly larger than those of both the GDX and GDX-E groups ($p < 0.001$, see Fig 3.1).

Barnes Maze Testing – No Drug Infusion:

Task Acquisition: Day 1 Testing. During Trial 1, on the first day of testing, all groups showed similar, variable levels of performance. Thus, regardless of sex or hormone status, it took rats fewer than 10 sec to initiate hole searches but from 1-2 min to locate the goal (Fig 3.2). The total path lengths used in explorations were also lengthy and ranged from roughly 250 to 350 cm; for most groups one third or less of the path length involved traversing the maze center (Fig 3.3). Finally, in locating the goal, rats investigated an average of 8-18 incorrect locations. For most groups, this included substantial numbers of re-investigations of incorrect sites (Fig 3.4).

Over the subsequent three trials, latencies to initiate search and locate the goals path lengths, and errors of all types progressively decreased in every group. These observations were borne out in a series of two-way ANOVAs with repeated measures designs that identified significant main effects of trial for all outcome measures except Primary Errors (Table 3.3) and in post hoc comparisons that identified significant differences in each outcome measure for trials 3 and/or 4 compared to trials 1 and 2 ($p = 0.0001$-0.05).

With the exception of Center Path Length, it was further evident that learning curves for all behavioral measures were relatively smooth, steep and overlapping for CTRL, GDX-E and GDX-TP rats, but were noticeably flatter for the GDX group. Thus, as trials proceeded and as all other groups improved, GDX rats continued to take longer

(Fig 3.2) and commit more errors (Fig 3.4) especially of the secondary and consecutive type in locating the goal, creating a divergence in the data that was especially evident over the final two trials. These differences were supported by two-way ANOVAs with repeated measures designs that identified significant main effects of Group for all measures except Center Path Length (Table 3.3), by post hoc comparisons that identified performance in the GDX group as significantly different from all others for every measure except Center Path Length ($p = 0.0001$-0.05).

Repeated Testing 24 h Later: Day 2 Resting. Four trials were conducted on Day 2, 24 h after Day 1 acquisition trials. Retention of the goal location was evident in all groups in performance in Trial 1 that equaled or bettered that recoded on the final trial from the previous day. This was confirmed in within-groups comparisons (paired t-tests) that found no significant differences in Day 1/Trial 4 compared to Day 2/Trial 1 data for any group on any outcome measure. Day 2 performance was also largely overlapping and asymptotic for all groups; although some variance was seen in the FEM group for path length and error measures (Fig 3.3A, B; 3.4A, B), for the most part, similar levels of peak performance were rapidly achieved and sustained for all groups. This was supported by a series of two-way ANOVAs with repeated measures design that found no significant or near-significant main effects of Group or Trial and no significant or near-significant interactions between these two for any performance measure.

One-Week Retention Trial: Day 7 Testing. A single retention trial was conducted one week after task acquisition. For all groups latencies to initiate search and to locate the goal were similar to each other and similar to the peak performance observed one week prior on Testing Day 2. This was confirmed in within-groups comparisons that found no significant differences in Day 1/Trial 4 compared to Day 2/Trial 1 data for these measures in any of these groups. For all but the GDX rats, path length measures (Fig 3.3) and numbers of errors (all types, Fig 3.4) were largely overlapping across groups are were not significantly different (paired t-tests) from testing Day 2 values. In contrast, the GDX group made more errors (all types, Fig 3.4) and followed longer path lengths (all types, Fig 3.2) in locating the goal compared to the other groups. Although these group differences were not significant, within-groups comparisons identified significant differences within the GDX cohort for Day 2 vs. Day 7 measures of total, primary and secondary errors [$t_{(7)}$ =2.35-3.67, p = 0.008-0.05] and measures of total and peripheral maze path lengths [$t_{(7)}$ =3.46-5.70, p =0.001-0.011] (Fig 3.4).

Maze Solving Strategy: Traces of animals' pathways and quantitative assessments of the sequences of their hole searches were used to characterize solving strategies as either random, serial, or spatially constrained/direct. All groups began testing using searches that were random. The FEM group was distinctive in rapidly replacing the random approach to one wherein the edge of the maze and thigmotaxis were used to serially investigate holes (Figs 3.5, 3.6). In contrast, the CTRL and GDX-TP rats developed serial (non-thigmotactic) and finally spatially limited/direct approaches to the locating the goal (Figs 3.5, 3.6). Finally, the GDX and GDX-E rats

also progressed from an initially random to more systematic, spatially focused searches. However, it was not until the final few trials on Day 2 that GDX and GDX-E rats began to reliably navigate more directly to the goal location (Figs 3.5, 3.6). The observed transitioning from group-wide utilization of random to group specific use of serial and/or direct modes of search strategy across trials was supported in statistical assessments (Fishers Exact text) that progressively approached and reached significance ($p = 0.001$) by Day 1 trial 4.

Barnes Maze Testing – Drug Infusion:

Task Acquisition: Day 1 Testing. On Day 1, Trial 1, all groups in both the saline and APV infusion cohorts showed similar, variable levels of performance. Thus, regardless of drug infusion or hormone status, it took rats fewer than 7 sec to initiate hole searches but from 1-2 min to locate the goal (Fig 3.7). Further, in locating the goal, rats investigated an average of 9-20 incorrect locations and for most groups, this included substantial numbers of re-investigations of incorrect sites (Fig 3.8).

Over the subsequent three trials, latencies to initiate search and locate the goals and errors of all types progressively decreased in every group of both the saline and APV treatment cohorts. These observations were borne out in a series of two-way ANOVAs with repeated measures designs that identified significant or near significant main effects of trial and/or group for all outcome measures (Tables 3.4,3.5).

In saline infused rats, it was further evident that learning curves were relatively smooth for each measure, overlapping for CTRL, GDX-E and GDX-TP rats, but were noticeably flatter for the GDX group, similar to the observations described above for the

no infusion cohort. Thus, as trials proceeded and as all other groups improved, GDX rats continued to take longer (Fig 3.7A,B) and commit more errors (Fig 8) in locating the goal. These differences were supported by two-way ANOVAs with repeated measures designs that identified significant main effects of Group for all measures except secondary and consecutive errors (Table 3.4), post hoc comparisons identified performance in the GDX group as the only one to differ from CTRL for each of these measures ($p = 0.001$-0.079) (Figs 3.7-3.8).

Conversely, in the APV infused cohort, GDX rats displayed steep learning curves across most measures while curves for CTRL, GDX-E, and GDX-TP rats remained comparatively flatter across the final three trials of Day 1. Thus, while GDX rats showed steady improvement, CTRL, GDX-E, and GDX-TP rats continued to take longer (Fig 3.7C,D) and commit more errors (Fig 3.8). These differences were supported by two-way ANOVAs with repeated measures designs that identified significant main effects of Group for all measures except latency to search (Table 3.5), and by post hoc comparisons that identified performance in the GDX group as the only one to significantly or near significantly differ from that of CTRLs ($p = 0.002$-0.083)(Figs 3.7-3.8).

Repeated Testing 24 h Later: Day 2 Resting. Four trials were conducted on Day 2, 24 h after Day 1 acquisition trials. Retention of the goal location was evident in all groups of both infusion cohorts in performance in Trial 1 that equaled or bettered that recoded on the final trial from the previous day. This was confirmed in within-groups comparisons (paired t-tests) that found no significant differences in Day 1/Trial 4

compared to Day 2/Trial 1 data for any group on any outcome measure. Day 2 performance was also largely overlapping and asymptotic for all groups; similar levels of peak performance were rapidly achieved and sustained for all groups and cohorts. This was supported by a series of two-way ANOVAs with repeated measures design that found no significant or near-significant main effects of Group or Trial and no significant or near-significant interactions between these two for any performance measure.

DISCUSSION

Sex differences and activational hormone effects have been identified for diverse aspects of learning, memory, and cognition in humans and animals (Christiansen and Knussmann, 1987; van Haaren et al., 1990; Gouchie and Kimura, 1991; Dohanich, 2002; Daniel et al., 2003; Luine, 2007). While spatial cognition and spatial learning appear to be sensitive to both biological sex and sex hormones in humans (Linn and Petersen, 1985; Hampson, 1990b; Voyer et al., 1995; Astur et al., 2004; Driscoll et al., 2005), the literature, while generally supportive of a male advantage in human and in animal models alike, is not completely consistent. For animal studies, differences in the duration of hormone deprivation, the doses of hormone replacement, the paradigms used in behavioral testing and in the mnemonic constructs measured are likely to contribute to study-to-study differences (Galea et al., 2001; Dohanich, 2002; Jonasson, 2005; Daniel et al., 2006; Sandstrom et al., 2006; Spritzer et al., 2008; Dohanich et al., 2009; Spritzer et al., 2011). More recently, however, specific attention has been paid to the sex-specific sensitivity of outcome measures to task-related factors of stress and

reward contingencies (McConnell et al., 2012). Building on recent studies using an object in place spatial memory task (McConnell et al., 2012), the studies presented here used non-rewarded, relatively stress-neutral conditions of Barnes maze testing (Harrison et al., 2009). This task enabled us to compare multiple aspects of spatial cognition, e.g. spatial learning, working and reference memory, in gonadally intact male, gonadally intact female, GDX male, and in GDX male rats supplemented with TP or E as well as the effect of NMDA antagonists on these behaviors in the male groups. The group differences that are discussed further below are first demonstrations of such effects using Barnes maze testing. They also confirm and extend demonstrations of sex hormone impact on spatial cognition under testing conditions that deliberately minimize other potentially confounding, hormone-sensitive non-mnemonic influences on behavior (McConnell et al., 2012).

Effects of GDX in Adult Male Rats: Task Acquisition, Spatial Learning and Spatial Working Memory. In Barnes maze testing, rats progressively navigate the apparatus using shorter, more direct routes to the goal while exploring fewer and fewer incorrect hole locations along the way. During Day 1 testing, CTRL (gonadally intact male) rats acquired the task rapidly as evinced by trial-to-trial reductions in all outcome measures. This improvement was accompanied by a shift in search strategy from random to more organized serial investigations of adjacent holes that were closer and closer to the goal and by the final trials, most CTRL rats navigated directly to the goal. Evidence of patent spatial working memory was also reflected in the group's diminishing number of primary errors and near elimination of secondary and consecutive type errors. In

contrast, GDX rats showed less improvement across Day 1 trials and continued to commit relatively high numbers of secondary and consecutive type errors. The GDX rats also persisted in random hole searches. These deficits resembled those reported in earlier studies using other spatial mazes and paradigms. For example, GDX-induced deficits in task acquisition have been shown for T-maze delayed alternation (Kritzer et al., 2001), radial arm maze (Daniel et al., 2003) and operant delayed spatial alternation tasks (van Hest et al., 1988; Kritzer et al., 2007). Similar to their commission of secondary and consecutive Barnes maze errors, GDX rats have also been shown to re-enter previously visited arms significantly more often than controls in radial arm maze tasks (Gibbs and Johnson, 2008; Spritzer et al., 2008; Hasegawa and Mochizuki, 2009) and show impaired retention in a delayed match-to place version of the Morris water maze (Sandstrom et al., 2006). However, a critical difference is that the present studies demonstrate GDX-induced deficits under non-rewarded contingencies whereas earlier evidence of GDX-induced acquisition and spatial working memory deficits were gleaned from behaviors motivated by either food (Hasegawa and Mochizuki, 2009), palatable food (Daniel et al., 2003; Spritzer et al., 2008), or by food or water under conditions of food or water restriction (Kritzer et al., 2007; Gibbs and Johnson, 2008). This is significant as GDX rats show significantly reduced sucrose preference (Carrier and Kabbaj, 2012), increased novelty–induce hypophagia (Carrier and Kabbaj, 2012), lower rewarded response rates in operant random ratio acquisition tasks (Heinsbroek et al., 1987) and earlier break-points in operant progressive reward ratio tasks (Kritzer et al., 2007) compared to controls. Differences in stress associated with the different tasks may also be relevant given that GDX rats show greater anxiety and greater behavioral

impairment in response to stress and circulating stress hormones (Osborne et al., 2009; Carrier and Kabbaj, 2012; Khakpai, 2014) compared to controls. With GDX effects on stress response and reward sensitivity poised to contribute to the poor performance reported among GDX groups in earlier studies, it is important that two studies have now shown significant GDX-induced impairment in spatial working memory under testing conditions wherein reward contingencies and stressors were relatively mitigated (McConnell et al., 2012). Further, by using the Barnes maze protocol, additional aspects of spatial cognition were also measured from behaviors motivated by rats' preferences for dark places. Given that dark preference has been shown to be potentiated by GDX in male rats compared to controls (Carrier and Kabbaj, 2012), the present study not only confirms and extends findings of GDX-induced deficits on spatial cognition but also shows them to be robust to testing conditions where motivation to perform the task may in fact be *enhanced* in GDX rats relative to CTRLS.

Effects of Hormone Replacement in Adult Male GDX Rats: Acquisition, Spatial Learning and Spatial Working Memory. The GDX-induced behavioral deficits observed during Barnes maze Day 1 testing were fully attenuated by giving GDX rats either E or TP. Thus, across all trials, latencies to find the goal, path lengths and numbers of primary, secondary, and consecutive type errors were overlapping in the CTRL, GDX-TP, and GDX-E groups. The comparable effects of supplementing GDX rats with TP or E suggest that the significant behavioral deficits induced in all of these domains by GDX stem at least in part from a loss of estrogen and/or estrogen metabolites. These findings contradict earlier studies from this lab where GDX-induced deficits in

acquisition of T-maze (Kritzer et al., 2001) and operant spatial lever press tasks (Kritzer et al., 2007) were attenuated in GDX rats supplemented with TP but not with E. Although the dose and duration of hormone replacement have been shown to impact spatial cognition in GDX and gonadally intact male rats (Goudsmit et al., 1990; Clark et al., 1995; Gibbs, 2005; Spritzer et al., 2013), these factors were similar across studies and are thus unlikely to underlie the disparities in outcomes. Similarly, while Barnes maze testing has been shown to have minimal effects on rats' cortisol levels (Harrison et al., 2009), rats in the prior studies were acclimated to handling, apparatus, and were subject to weeks-long testing, suggesting that cross-study differences in animals' stress responses may also have been minimal. In contrast, differences in reward contingencies do parse along lines of the effectiveness of TP vs. E-replacement to attenuate behavioral deficits in GDX rats. Thus, in both the T-maze and operant tasks -- where replacing GDX rats with TP but not E replacement effectively rescued GDX-induced acquisition deficits, subjects worked for water reward under similar protocols for water restriction (Kritzer et al., 2001; Kritzer et al., 2007). However, in the present study where GDX-induced deficits in acquisition and spatial cognition were equally and fully attenuated by both TP and E, all measured behaviors were spontaneous. These findings have parallels in GDX-induced deficits observed in a non-rewarded, object location memory paradigm which were also shown to be attenuated by supplementing GDX rats with E, T, or with dihydrotestosterone (DHT) (McConnell et al., 2012). Because DHT can be metabolized to estrogen receptor agonist compounds (3α and 3β-diol derivatives) (Jin and Penning, 2001; Torn et al., 2003; Wang et al., 2009), the data may suggest selective benefits of androgenic over estrogenic replacement in enhancing

motivation to acquire tasks, and benefits of estrogenic over androgenic replacement in stimulating spatial cognition per se. Such a division of labor fits with evidence for reinforcing properties of androgens (Wood, 2004), and underscores the need for further assessment using paradigms that can effectively isolate the influences of gonadal steroids on cognitive, mnemonic, and non-mnemonic elements of the task.

Effects of GDX and Hormone Replacement in Adult Male Rats: Task Retention and Spatial Reference Memory. In contrast to the significant negative effects of GDX on Barnes task acquisition and spatial working memory, only subtle, non-significant GDX effects were observed in behaviors measured during retention testing 24 h later. Thus, while the GDX and GDX-E groups showed some initial improvement during Day 2 testing, asymptotic, peak performance that was similar in all groups emerged within a trial or two. Interestingly, the non-significant 'catch-up' observed in the GDX and GDX-E groups coincided with their transitioning from random to serial to spatially focused, direct navigations to the goal —similar to the transitions observed in the CTRL and GDX-TP groups during the first few acquisition trials. When tested on a single trial one week later, it was also evident that all groups retained the task. However, GDX rats made more errors of all types than the other groups, and significantly more errors than this same group made on the final trial of Day 2 testing.

Findings of no group differences in Barnes maze retention testing at a 24 h and 1 week delay add to consensus views of minimal to no effects of GDX on spatial reference memory. However, findings that after a week interval GDX rats made significantly more errors and followed longer pathways significantly more than they had

previously does suggest some GDX influence on spatial reference memory. This may be in line with findings that T-replacement in GDX rats improves and T administration in gonadally intact rats impairs Morris Water maze spatial reference memory in adult but not aged rats (Goudsmit et al., 1990; Khalil et al., 2005; Spritzer et al., 2011). Together with the present findings, these data underscore the potential complexity of hormone effects on spatial reference memory in male rats and suggest that rigorous experimental control and perhaps more demanding memory tasks may be necessary to fully characterize them.

Sex and Sex Hormone Effects on Barnes Maze Strategies in Adult Male and Female Rats.

All female subjects rapidly adopted and utilized a thigmotactic serial search strategy; within one or two trials nearly every member of the group followed the outer edge of the maze to investigate each hole encountered until arriving at the goal. These strategies had distinctive proprioceptive elements; subjects consistently followed the maze edge in either the clockwise or counter clockwise direction, and did not deviate in terms of approach or preferred direction across trials or testing days. These observations in the Barnes maze are thus commensurate with a female preference for response strategies in solving spatial mazes (Kanit et al., 2000; Jonasson, 2005; Blokland et al., 2006; Hawley et al., 2012).

The thigmotactic response strategies used by the females contrasted sharply with the spatial navigation that eventually was used by all male groups. This made it difficult to decipher comparative meaning from many of the other performance

measures. For example, although most other behaviors measured, e.g., latency to find goal, commission of secondary and consecutive type errors, were similar in CTRL and female rats, it is far from clear that these reflect group similarities in task acquisition, spatial working, or spatial reference memory. In fact, those instances where scores between males and females diverged, i.e., the increased commission of primary errors and longer peripheral maze path lengths followed by females, suggest that their preferred spatial solving strategies enable them to locate the goal without tapping spatial working memory. Thus, the Barnes maze holds important advantages in meeting necessary challenges of parsing the effects of gonadal steroids on stress response and motivation to advance understanding of their impact on spatial cognition (Luine, 2002; Bowman et al., 2003; Bowman et al., 2009; ter Horst et al., 2012; McHenry et al., 2014). However, the degrees of freedom with which animals can solve the task may complicate the resolution of sex differences among behavioral domains of spatial working memory.

Effects of APV on Barnes Maze Performance in Adult Male, GDX, GDX-E, and GDX-TP Rats

Rats receiving infusions of saline showed expected performances on the Barnes maze. Thus, while CTRL, GDX-TP, and GDX-E rats showed steady improvements across the trials of Day 1, GDX rats displayed little improvement. Conversely, the rats receiving infusions of APV showed a reversal of these group trends such that the GDX rats showed steady improvement across Day 1 trials, while CTRL, GDX-E, and GDX-TP rats displayed flat learning curves across all measures.

Previous studies have shown that GDX elevates PFC DA levels in an androgen sensitive, estrogen insensitive manner (Aubele and Kritzer, 2011) and the work presented in Chapter II found GDX-induced increases in both firing rates and bursting activity of DAergic VTA cells. Further, microdialysis studies have found that administration of NMDA antagonists into the PFC reduces the elevated PFC DA levels found in GDX rats and increases the basal DA levels in CTRL rats (Aubele and Kritzer, 2012). Additionally, as presented in Chapter II, electrophysiology studies have shown that NMDA antagonist infusion into the PFC increases the firing rates of DAergic VTA cells in CTRL rats and decreases them in GDX rats. Thus, as shown here, while GDX induces deficits in spatial working memory that coincide with elevations in basal PFC DA levels, the neurochemical and physiological changes that result following PFC NMDA antagonism appear to rescue the behavioral deficits in these animals. Conversely, the NMDA antagonist induced increases in both firing rates and PFC DA levels appear to induce deficits in spatial working memory of CTRL rats. These effects appear to be androgen sensitive, as the neurochemical, physiological, and behavioral effects of GDX are rescued in GDX-TP rats. Less clear, however, are the effects of E, which rescues behavior but not the GDX induced elevations in PFC DA levels or DAergic cell firing rates. As discussed above, like androgen, estrogen may play a role in mediating executive functions, which, taking these results into account, may lie outside of the DAergic circuitry discussed here.

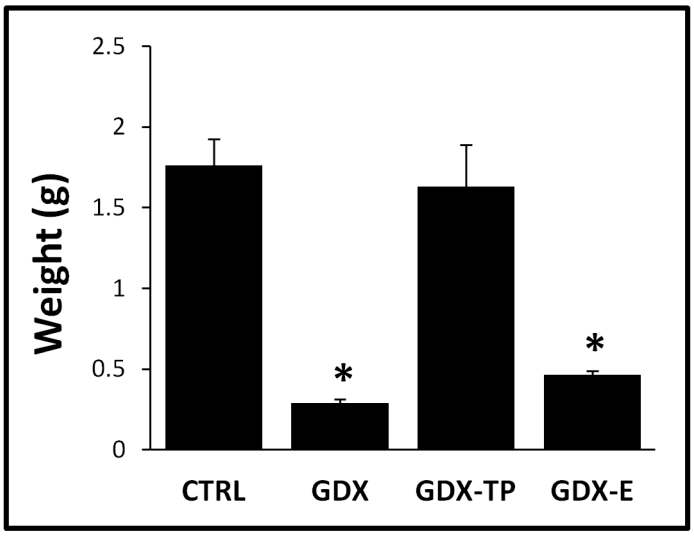

Figure 3.1. Bar graphs showing average bulbospongiosus muscle weights in grams (g) for gonadally intact control (CTRL), gonadectomized (GDX), and gonadectomized male rats supplemented with testosterone propionate (GDX-TP) or estradiol (GDX-E). Muscle weights of CTRL and GDX-TP rats were similar to each other and were significantly greater than those of GDX and GDX-E rats. Muscle weights of GDX and GDX-E rats were also similar to each other. Error bars represent standard errors of the mean. Asterisks denote significant differences from CTRL for post-hoc testing at the $p < 0.05$ level.

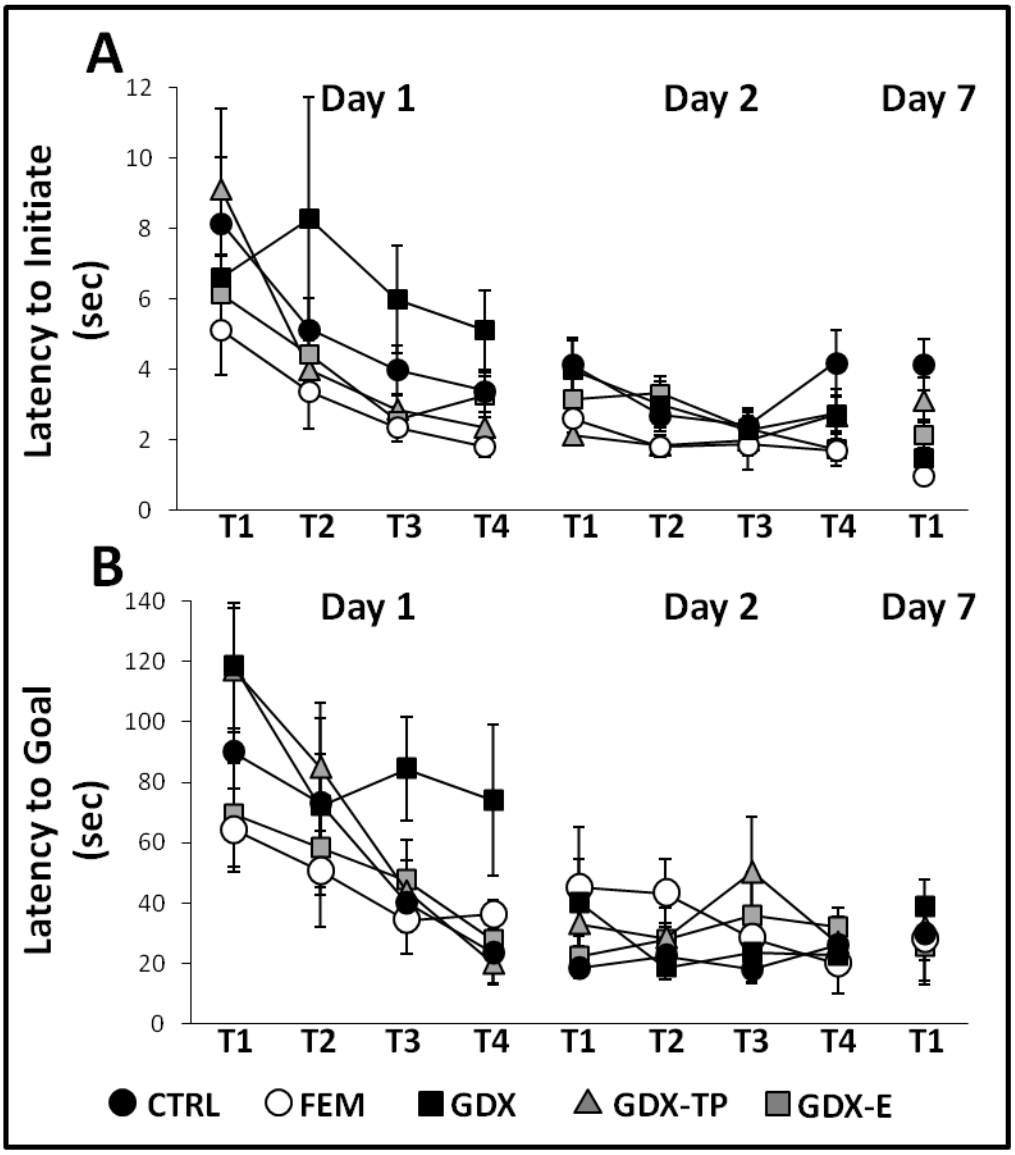

Figure 3.2. Line graphs showing (A) average latencies to initiate search and (B) average latencies to locate the goal across the four trials (T1-T4) of Day 1 (acquisition), the four trials of Day 2 (retention), and single trial of Day 7 (retention) testing for females (FEM, white circles), gonadally intact control (CTRL, black circles), gonadectomized (GDX, black squares), and gonadectomized male rats supplemented with testosterone propionate (GDX-TP (gray triangles) or estradiol (GDX-E, gray squares). Error bars represent standard errors of the mean. For all but the GDX group, latencies measured on Day 1 were similar and decreased systematically across trials. In the GDX rats, latencies tended to be longer than those of the other groups and were most markedly longer on Trials 3 and 4. During Day 2 and Day 7 testing, latencies were consistent and short across all trials in all groups.

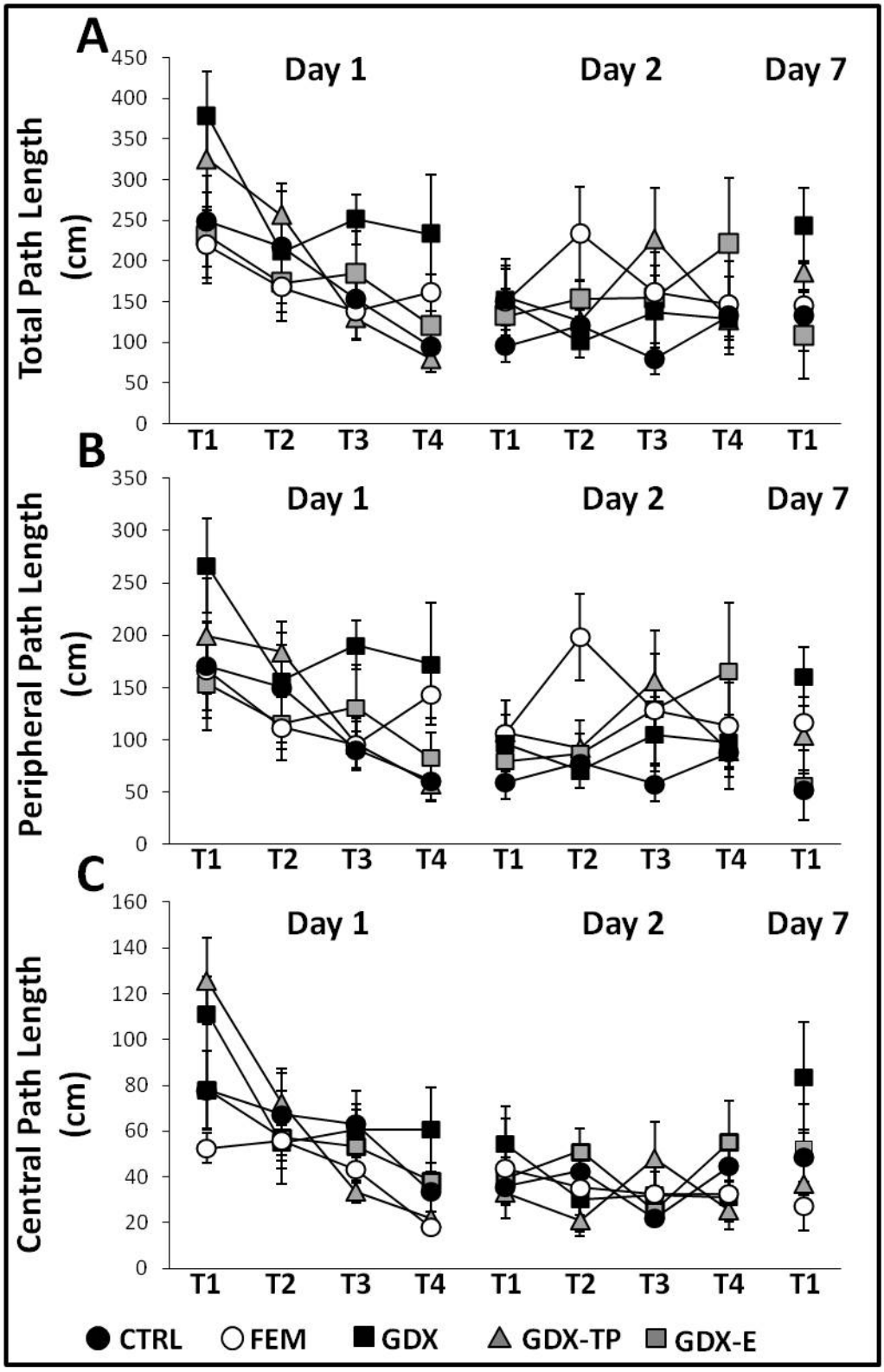
A
Total Path Length (cm)
Day 1
Day 2
Day 7
450
400
350
300
250
200
150
100
50
0
T1 T2 T3 T4 T1 T2 T3 T4 T1
B
Peripheral Path Length (cm)
Day 1
Day 2
Day 7
350
300
250
200
150
100
50
0
T1 T2 T3 T4 T1 T2 T3 T4 T1
C
Central Path Length (cm)
Day 1
Day 2
Day 7
160
140
120
100
80
60
40
20
0
T1 T2 T3 T4 T1 T2 T3 T4 T1
CTRL
FEM
GDX
GDX-TP
GDX-E

Figure 3.3. Line graphs showing (A) average total path lengths, (B) average peripheral maze path lengths and (C) average central maze path lengths -- all measured in centimeters (cm), during the four trials (T1-T4) of Day 1 (acquisition), the four trials of Day 2 (retention), and single trial of Day 7 (retention) testing for females (FEM, white circles), gonadally intact control (CTRL, black circles), gonadectomized (GDX, black squares), and gonadectomized male rats supplemented with testosterone propionate (GDX-TP, gray triangles) or estradiol (GDX-E, gray squares). Error bars represent standard errors of the mean. On Day 1 testing, all but the GDX and FEM groups showed similar, consistently shortened (A) total, (B) peripheral, and (C) center maze path lengths across trials. The GDX rats (black squares) on all measures of path length showed relatively small decreases across trials and were noticeably longer than those of all other groups, especially for T3 and/or T4. The FEM rats (white circles) were distinguished by relatively long peripheral maze paths (B) that were most noticeable in T4. During Day 2 and Day 7 testing, all path length metrics were similar and consistently short across trials in all groups.

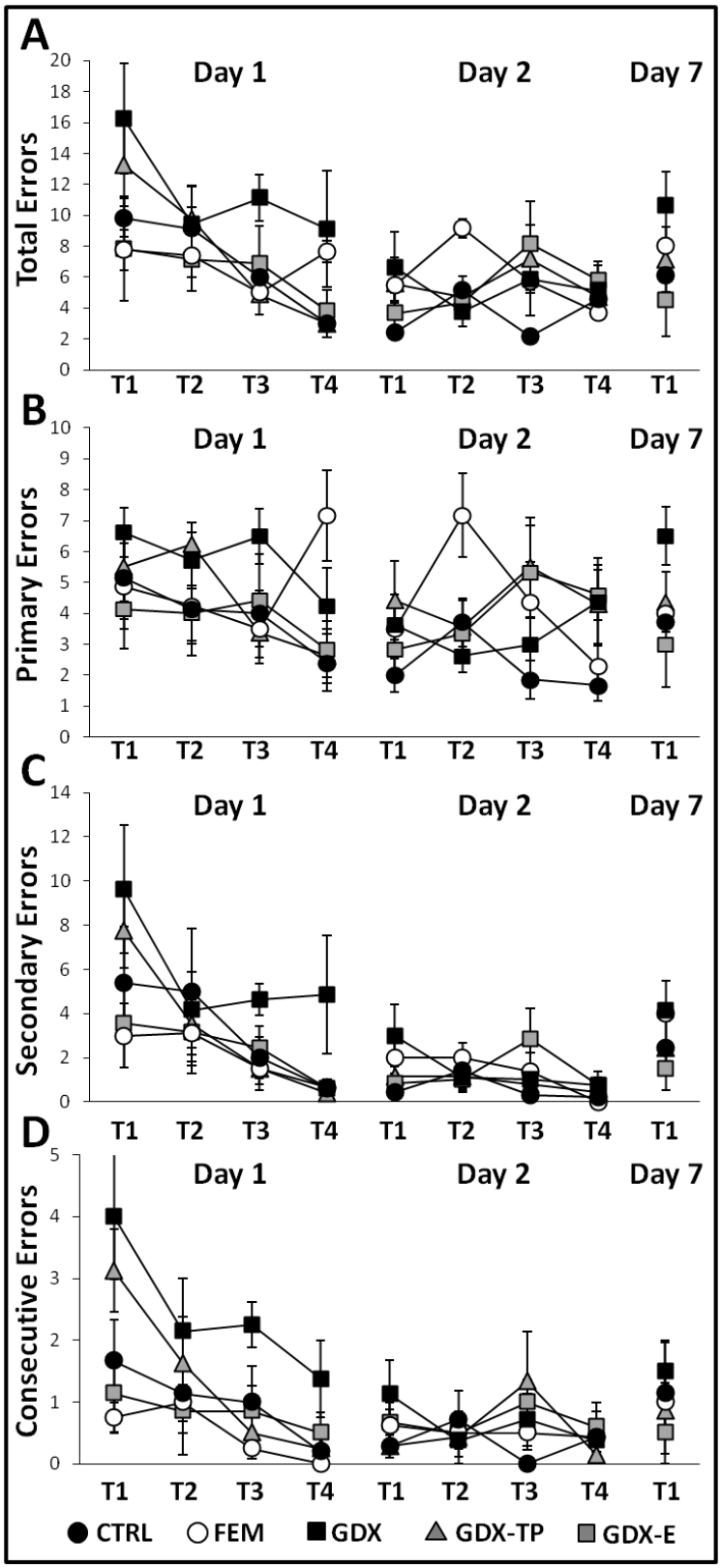
A
Total Errors
Day 1
Day 2
Day 7
T1 T2 T3 T4 T1 T2 T3 T4 T1
B
Primary Errors
C
Secondary Errors
D
Consecutive Errors
CTRL
FEM
GDX
GDX-TP
GDX-E

Figure 3.4. Line graphs showing (A) average numbers of total errors, (B) average numbers of primary errors, (C) average numbers of secondary errors, and (D) average numbers of consecutive errors committed during the four trials (T1-T4) of Day 1 (acquisition), the four trials of Day 2 (retention), and the single trial of Day 7 (retention) testing for females (FEM, white circles), gonadally intact control (CTRL, black circles), gonadectomized (GDX, black squares), and gonadectomized male rats supplemented with testosterone propionate (GDX-TP, gray triangles) or estradiol (GDX-E, gray squares). Error bars represent standard errors of the mean. For all but the GDX group, total (A) and primary (B) errors made during Day 1 testing were similar and decreased consistently across trials; the numbers of secondary (C) and consecutive errors (D) were also similar, were lower than primary errors, and dropped to near zero levels by T3 and T4. The GDX rats (black squares) made more errors of all types, and notably secondary and consecutive types, compared to all other groups, whereas the FEM rats (white circles) were distinguished by greater commissions of primary errors than the other groups. During Day 2 testing, error rates of all kinds were similar and consistently low across trials in all groups. On Day 7 testing, though no group differences were found, GDX rats (black squares) made significantly more total, primary, and secondary errors compared to T4 of Day 2 testing.

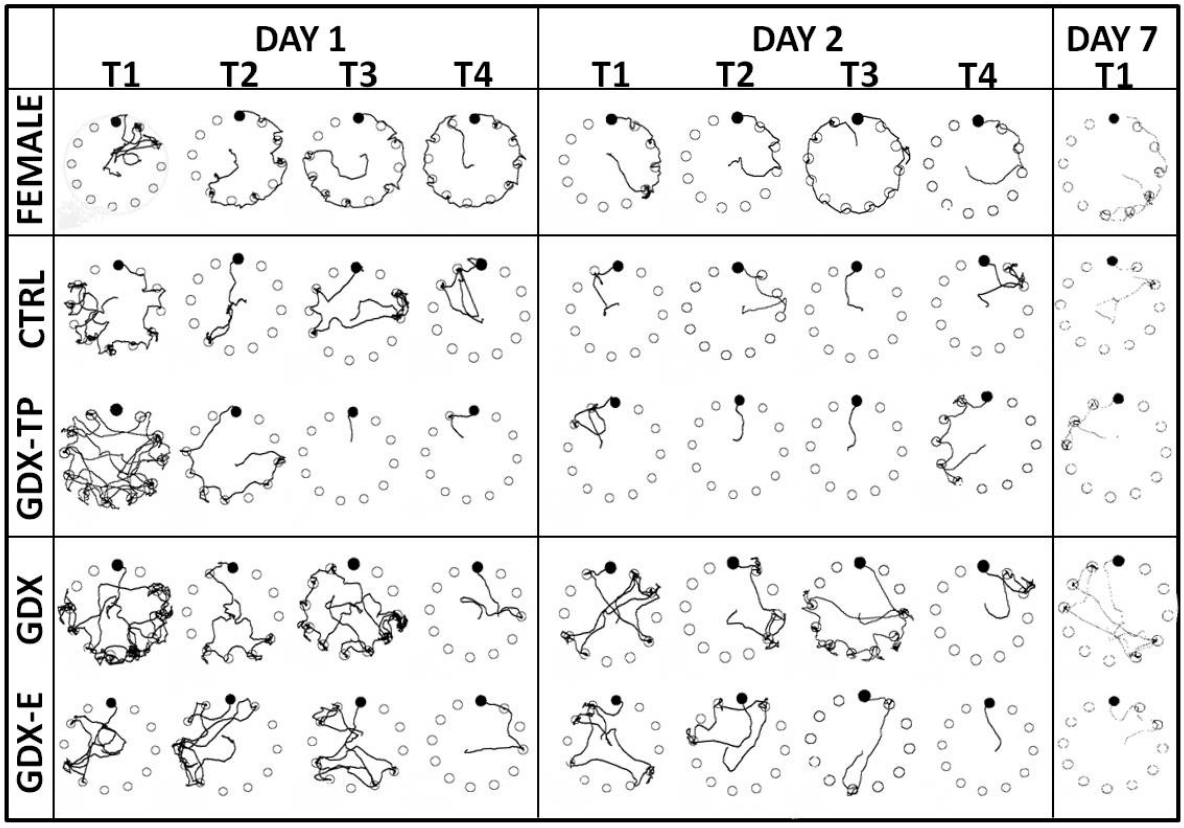

Figure 3.5. Digitized traces of paths followed by representative group subjects across Day 1, Day 2, and Day 7 trials. Open circles mark maze holes and the black circle marks the goal location. The traces illustrate the unique serial, thigmotactic hole investigation strategy that was consistently used by female rats; animals were remarkably invariant in following the physical edge of the maze in either a clockwise or counterclockwise direction to locate the goal. In contrast, control (CTRL) rats and gonadectomized rats supplemented with testosterone propionate (GDX-TP) rapidly transitioned across trials from random to serial to direct navigational strategies, while GDX and GDX rats supplemented with estradiol (GDX-E) rats needed a second testing day to transition from random to more systematic and spatially localized means to locate the goal. On Day 7 testing, female, CTRL, GDX-TP, GDX-E rats retained their strategies while GDX rats showed slightly less navigational retention.

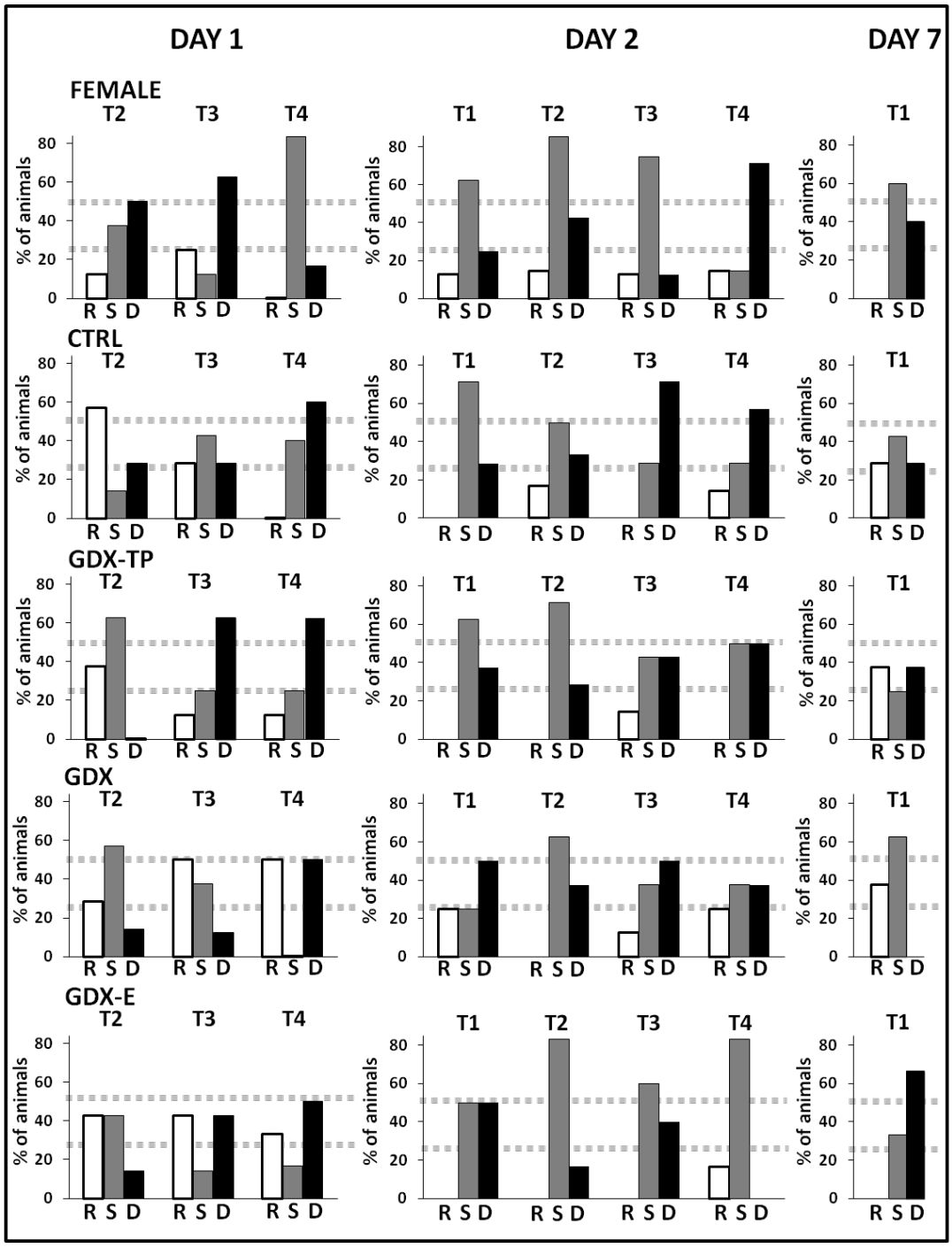

Figure 3.6. Bar graphs depicting percentage of animals utilizing random, serial, or direct searches for the goal across the last three trials (T2-T4) of Day 1 (acquisition), the four trials of Day 2 (retention), and the single trial of Day 7 (retention) testing. From T2 to T4 on Day 1, the percentage of control male rats (CTRL) and gonadectomized male rats supplemented with testosterone propionate (GDX-TP) utilizing primarily random

searches (white bars) consistently decreased while those using serial (gray bars) or direct (black bars) searches progressively increased. By T4, the majority of CTRL and GDX-TP rats were moving directly to the goal with some rats continuing to employ serial searches and few to none performing random searches. Conversely, a large number of gonadectomized rats (GDX) and gonadectomized rats supplemented with estradiol (GDX-E) continued to demonstrate random searches from T2 through T4 with the percentage of animals doing so showing no appreciable decrease across acquisition trials. Additionally, while the number of GDX and GDX-E rats using direct searches progressively increased across trials and the number of rats performing serial searches decreased, the overall number of rats employing either of these types of searches did not change from T2 to T4. Finally, from T2 to T4, very few female rats demonstrated random searches. Most female rats used either serial or direct searches for the goal with the majority performing serial searches by T4. Across Day 2 trials, the majority of CTRL, GDX-TP, GDX, and GDX-E rats utilized either serial or direct searches with very few animals using a random search strategy while females continued to prefer serial searches. On Day 7, female and GDX-E rats maintained their navigational strategies while CTRL, GDX-TP, and GDX rats showed less retention with more rats using random searches compared to other groups. Fisher's exact tests only reached significance on Day 1, Trial 4 ($p < 0.01$).

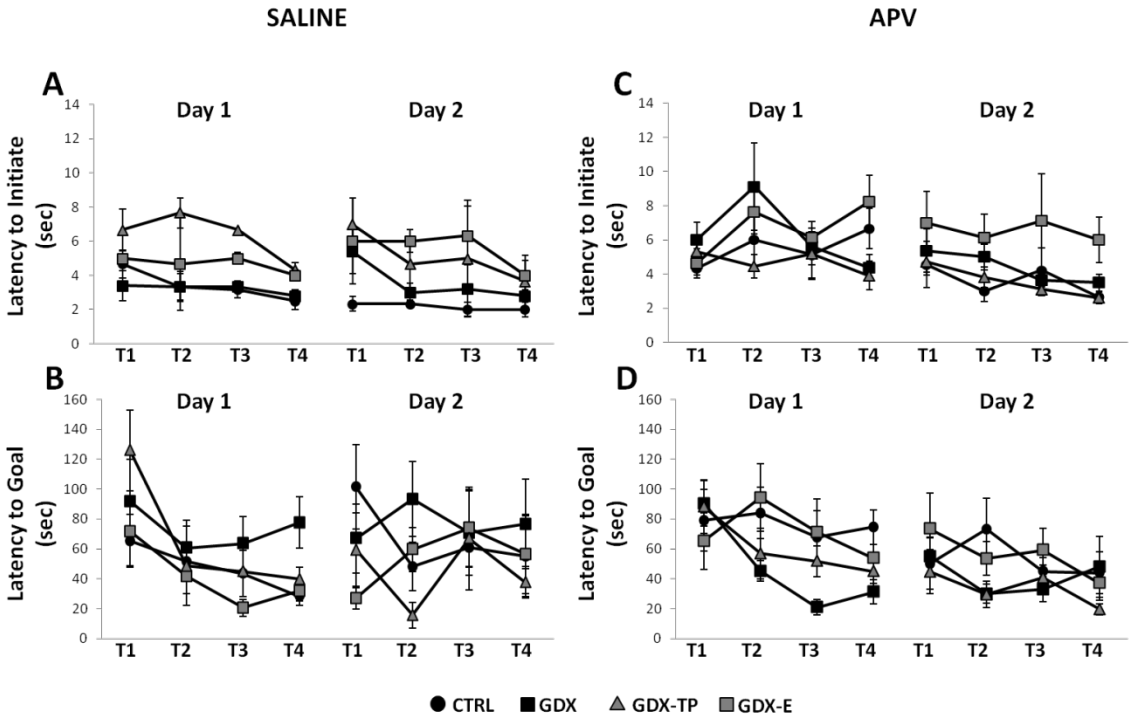

Figure 3.7. Line graphs depicting latencies to initiate search and latencies to goal in saline and APV treated rats. Though no differences were observed in the latencies to initiate search (A,C), latencies to goal did parse out drug and hormone-treatment effects (B,D). Thus, in saline treated rats (B), all groups showed improvement across the four trials of Day 1, except for the GDX group, which did not improve past Trial 2. However, in APV treated rats (D), the GDX group improved much quicker compared to CTRL, GDX-TP, and GDX-E rats.

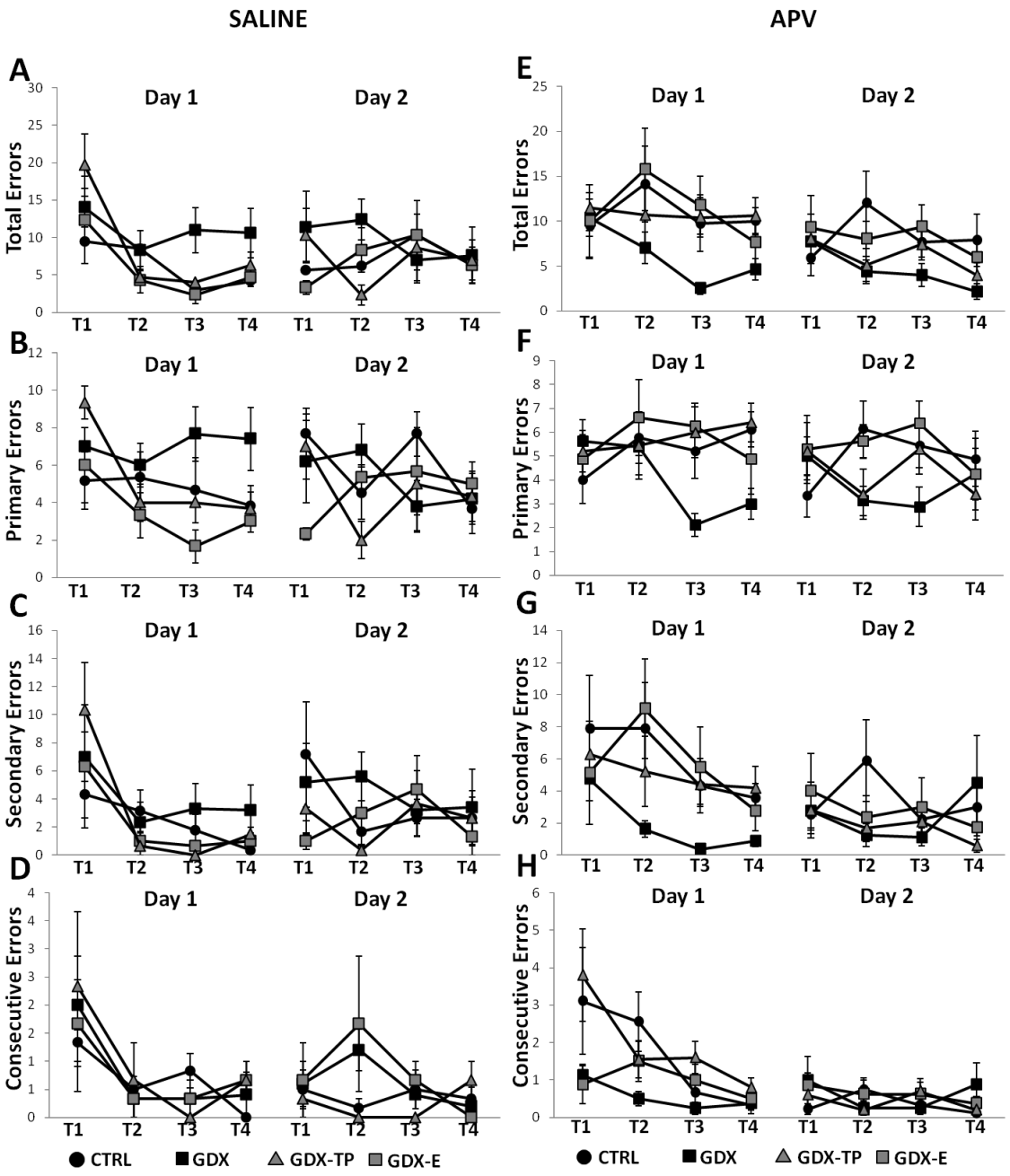

Figure 3.8. Line graphs depicting total, primary, secondary, and consecutive errors in saline and APV treated rats. Though no differences were observed in the consecutive errors (D,H), total, primary, and secondary did display drug and hormone-treatment effects. Thus, in saline treated rats (A-C), all groups showed improvement across the four trials of Day 1, except for the GDX group, which did not improve past Trial 2. However, in APV treated rats (E-G), the GDX group improved much quicker compared to CTRL, GDX-TP, and GDX-E rats.

Table 3.1
Number of rats in each estrous cycle stage across testing days as confirmed via vaginal lavage and vaginal cytology.

Cycle Stage	Testing Day 1	Testing Day 2	Testing Day 3
Estrus (low estrogen)	0	1	0
Diestrus	4	4[a]	5
Proestrus (high estrogen)	4	3[b]	3

[a] Two rats remained in diestrus from Day 1 to Day 2.

[b] One rat remained in proestrus from Day 1 to Day 2.

Table 3.2
Definitions of the quantitative measures of Barnes maze performance.

Outcome Measure	Units	Description
Latency to Initiate Search	Seconds	Total time between start cylinder removal and first hole investigation.
Total Errors	Number	Total number of incorrect hole location investigations per trial.
Primary Errors	Number	Total numbers of first-time investigations of incorrect holes per trial.
Secondary Errors	Number	Total numbers of reinvestigations of previously visited incorrect holes per trial.
Consecutive Errors	Number	Total numbers of consecutive reinvestigations of incorrect holes. Rats must have stepped away from the hole in between investigations.
Latency to Find Goal	Seconds	Total time between start cylinder removal and front paws touching down in the goal box.

Table 3.3
Results of 2-way analyses of variance (ANOVA) with repeated measures design for each measure across the four acquisition trials of Day 1. Significant or near significant main effects of group, trial, or group by trial interactions were found for each measure.

BEHAVIOR	MAIN EFFECTS OF GROUP	MAIN EFFECTS OF TRIAL	INTERACTIONS
Latency to search	($F_{4,33}$ = 2.43, p=0.07)	($F_{1.839,60.682}$ = 13.25, p<0.01)	NS
Latency to goal	($F_{4,33}$ = 4.76, p<0.01)	($F_{2.402,79.282,}$ = 11.64, p<0.01)	NS
Total path length	($F_{4,33}$ = 3.74, p=0.01)	($F_{3,99}$ = 10.70, p≤0.01)	NS
Peripheral path length	($F_{4,33}$ = 4.01, p=0.01)	($F_{2.229,73.573}$ = 6.49, p<0.01)	NS
Center path length	NS	($F_{2.425,80.038}$ = 16.65, p<0.01)	($F_{9.702,80.038}$=1.91,p=0.06)
Total errors	($F_{4.33}$ = 4.07, p=0.01)	($F_{2.169.71.574}$ = 5.23, p=0.01)	NS
Primary errors	($F_{4,33}$ = 3.08, p=0.03)	NS	NS
Secondary errors	($F_{4,33}$ = 4.05, p=0.01)	($F_{1.875,61.883}$ = 6.34, p<0.01)	NS
Consecutive errors	($F_{4,33}$ = 8.79, p<0.01)	($F_{2.002,66.069}$ = 9.32, p<0.01)	NS

NS: not significant

Table 3.4
Results of 2-way analyses of variance (ANOVA) with repeated measures design for each measure across the four acquisition trials of Day 1 in saline treated rats. Significant or near significant main effects of group, trial, or group by trial interactions were found for each measure.

BEHAVIOR	MAIN EFFECTS OF GROUP	MAIN EFFECTS OF TRIAL	INTERACTIONS
Latency to search	($F_{1,14}$ = 5.46, p=0.01)	($F_{3,39}$ = 7.68, p=0.03)	NS
Latency to goal	($F_{1,13}$ = 4.14, p=0.03)	($F_{2.033,26.435}$ = 5.69, p<0.01)	NS
Total errors	($F_{1,13}$ = 6.34, p<0.01)	($F_{3,39}$ = 7.43, p<0.01)	NS
Primary errors	($F_{1,13}$ = 10.35, p<0.01)	NS	NS
Secondary errors	NS	($F_{3,36}$ = 6.45, p<0.01)	NS
Consecutive errors	NS	($F_{3,39}$ = 9.65, p<0.01)	NS

NS: not significant

Table 3.5
Results of 2-way analyses of variance (ANOVA) with repeated measures design for each measure across the four acquisition trials of Day 1 in APV treated rats. Significant or near significant main effects of group, trial, or group by trial interactions were found for each measure.

BEHAVIOR	MAIN EFFECTS OF GROUP	MAIN EFFECTS OF TRIAL	INTERACTIONS
Latency to search	NS	($F_{2.297,71.194}$ = 2.325, p=0.098)	NS
Latency to goal	($F_{1,31}$ = 2.73, p=0.06)	($F_{2.515,77.970,}$ = 3.185, p=0.036)	NS
Total errors	($F_{1,30}$ = 3.87, p=0.02)	NS	NS
Primary errors	($F_{1,31}$ = 2.59, p=0.07)	NS	NS
Secondary errors	($F_{1,31}$ = 4.47, p=0.01)	($F_{2.343,72.645}$ = 2.46, p=0.08)	NS
Consecutive errors	($F_{1,31}$ = 4.10, p=0.02)	($F_{1.607,49.807}$ = 5.66, p=0.01)	NS

NS: not significant

General Discussion

Summary of Major Findings

The studies presented in this dissertation sought to explore the effects of sex and gonadal hormones on the regulation of PFC DA levels and PFC functions. The chapters of this dissertation asked 1) Do sex differences exist in the intraPFC regulation of PFC DA levels and do sex and gonadal hormones affect the locus of the NMDAR-mediated component of this regulation? 2) Do sex and sex hormones affect the firing patterns of VTA-projecting PFC pyramidal cells and/or the DAergic VTA cells they synapse onto? 3) How do sex and gonadal hormones affect PFC-dependent behaviors and can these behaviors be altered through NMDAR-targeted pharmacological interventions?

Though the intraPFC mechanisms modulating PFC DA levels have been well characterized in male rats, those in female rats have been remarkably understudied. Thus, in Chapter I, microdialysis studies sought to explore the GABAergic and GLUergic mechanisms regulating PFC DA tone across sex. Antagonists of GABA-A, GABA-B, AMPA, and NMDA receptors were administered via reverse dialysis while medial PFC DA levels were sampled and measured. The results showed that while GABA-A does not exert tonic control over PFC DA levels in either males or females, GABA-B exerts tonic inhibition in both, though to a much larger degree in females. Further, while AMPA receptor antagonists resulted in decreases of PFC DA levels in both sexes, NMDA receptor antagonists produced DA increases in males and decreases in females. Despite these regulatory differences, DA levels were similar across sex. Finally, the

hypothesis that the differences in NMDA-mediated DA regulation observed here in females and previously in GDX males is due to a shift in the locus of NMDA influence from inhibitory to excitatory sites was tested via dual drug challenge. Reverse dialysis infusion of a GABA-B antagonist was followed by co-infusion with an NMDA antagonist. In rats that showed increases in PFC DA levels following NMDA antagonist application alone, i.e. intact male and GDX-TP rats, effects of NMDA antagonists were blocked by co-infusion of the GABA-B antagonist, while in rats showing decreases in PFC DA levels following NMDA antagonism alone, i.e. female, GDX, and GDX-E rats, NMDA antagonists decreased PFC DA levels even with co-infusion of GABA-B antagonists. These findings provide support for the proposed shift in primary NMDA influence from inhibitory interneurons, as suggested by previous studies using males only, to downstream pyramidal cells in rats with low circulating androgen levels, i.e. female, GDX, and GDX-E rats.

Taking the findings presented in Chapter I into account, Chapter II explored the effects of sex and sex hormones on the physiology of the DA regulating circuitry as well as the NMDA-mediated influences on these cells. Thus, extracellular recordings of VTA-projecting pyramidal cells and DAergic VTA cells were performed in anesthetized rats. The results showed that while the cells of male, female, and GDX-TP rats displayed similar firing patterns, cells of GDX and GDX-E rats had significantly higher average firing rates and displayed significantly more bursting behavior, following the predictions made based on the findings of Chapter I. That cells of female rats did not show increases in firing rates lends evidence to the hypothesis that heightened GABA-B mediated inhibition keeps PFC pyramids in check. Additionally, following infusion of an

NMDA antagonist into the PFC, VTA DAergic cells were recorded in intact male and GDX rats. In intact males, firing rates increased, as did bursting activity, while both measures decreased in GDX rats, lending even further support to the hypothesis that low circulating androgen levels lead to elevated NMDA sensitivity and excitability of PFC pyramids.

The final chapter of this dissertation tested the effects of sex and sex hormones on DA-dependent PFC-mediated behaviors. Using the Barnes maze to test spatial working memory, I found that in many aspects, males and females perform to the same levels, but use drastically different search strategies. Additionally, GDX was shown to impair spatial working memory and both TP and, unexpectedly, E were shown to rescue this behavior to various degrees. When comparing spatial reference memory, all groups displayed similar levels of retention. Finally, these behaviors were tested following infusion of an NMDA antagonist into the PFC of male, GDX, GDX-E, and GDX-TP rats, which resulted in a reversal of the group trends. Thus, following drug infusion, GDX rats showed steady improvements across trials while CTRL, GDX-E, and GDX-TP rats showed little to no improvement on any measure. While the maze performances of male, female, GDX, and GDX-TP rats follow predictions based on neurochemical (Chapter I) and physiological (Chapter II) findings and point to strong androgen effects on this circuitry, GDX-E rats did not fully follow these predictions and the unexpected rescuing effects of E observed here suggest roles for estrogen as well. Overall, the work presented in Chapters I-III of this dissertation offers novel sex comparisons and provides strong evidence for androgen regulation of NMDAR-mediated modulation of PFC DA levels and DA-dependent PFC-mediated behaviors.

Future Directions

Gonadal Hormone Effects on the Prefrontal Cortex

The results of this dissertation show clear effects of gonadal hormones on the regulation of PFC DA tone and PFC-dependent behaviors. Particularly intriguing were the observed effects on the NMDAR-mediated influences on these functions, a clearer characterization of which would greatly benefit the understanding of this important circuit. The androgen effects on the NMDA sensitivity of PFC pyramids could potentially be explained by either upregulation of NMDA receptors, changes in NMDA receptor function, or both. Previous studies suggest that androgens may play a role in modulating both the quantity and function of NMDA receptors. For example, gonadectomy has been shown to increase NMDA antagonist binding in hippocampal pyramidal cells, an effect rescued by dihydrotestosterone supplement (Kus et al., 1995). Additionally, saturating levels of androgen receptor agonists have been found to affect hippocampal pyramidal cell physiology, causing increases in action potential duration and decreases in the amplitude of fast afterhyperpolarization (Pouliot et al., 1996). Saturating androgen levels were also found to protect these cells from NMDA-mediated excitotoxicity (Pouliot et al., 1996). Moreover, studies have shown that androgens can also modulate the subunit composition of NMDA receptors in hippocampal pyramidal cells (Le Greves et al., 1997). This last finding may be particularly relevant as the subunit composition of NMDA receptors can greatly alter channel kinetics, channel conductance, and sensitivity to Mg^{2+} block (Cull-Candy et al., 2001).

A number of techniques can be used to further describe the effects of androgens on the NMDA-mediated excitability of PFC pyramidal cells. First, intracellular recordings in PFC slices would elucidate the effects of androgens on NMDA-mediated pyramidal cell characteristics and physiology. In situ hybridization could be used to search for changes in subunit expression following hormone treatment and tissue fractionation and immunoblotting would shed light onto whether differences exist in receptor trafficking and anchoring.

Understanding how hormones affect the mechanisms behind changes in NMDA-mediated DA regulation is of particular importance clinically. Composition and function of NMDA receptors has been associated with many neurological diseases including schizophrenia (Javitt, 2010; Kantrowitz et al., 2010; Gonzalez-Burgos and Lewis, 2012), mood disorders (Mueller and Meador-Woodruff, 2004; Toro and Deakin, 2005; Beneyto and Meador-Woodruff, 2008; Feyissa et al., 2009), autism (Ghanizadeh, 2011; Carlson, 2012; Won et al., 2012), and Alzheimer's disease (Leuba et al., 2008), all of which differentially affect males and females. Understanding how hormones are altering the NMDA component of DA regulation and PFC function could lead to more efficient, effective, and sex-specific treatments in the future.

Sex Differences

While research on gonadal hormones and the brain has developed over a number of years to become an established field of neuroscience, neurobiological studies incorporating sex differences have remained low in numbers. This holds true despite clear sex differences in both health and disease. For example, men are more

likely to be diagnosed with attention deficit hyperactivity disorder, dyslexia, autism, and schizophrenia while women are more often diagnosed with major depressive disorder, anxiety, panic disorders, and anorexia (McCarthy et al., 2012). However, sex differences remain understudied. To demonstrate, a search for "female prefrontal cortex" in Pubmed shows that the number of studies per year on this topic did not exceed 100 until 1995 and remained below 1000 per year until 2008. To compare, a search for "male prefrontal cortex" resulted in over twice as many results in 1995 and nearly 50 percent more in 2008. Further, the first study of sex differences in the prefrontal cortex was not published until 1984 and there were less than 100 studies per year on the topic even as recently as 2013. In 2010, Beery and Zucker (2011) found that the field of neuroscience was the worst offender for male over female preference in experimental animal research with male-only research outnumbering female studies 5.5:1. In light of so few studies despite such pervasive sex differences in some of the most detrimental neurological diseases, in 2011 the National Institutes of Mental Health convened a workshop to discuss the paucity of sex difference research in neuroscience, the extensiveness of sex differences in the brain, and the need for more scientists to incorporate both sexes into future studies (Pankevich et al., 2011). Attendees from a number of disciplines collectively concluded that strong impetus to study both sexes exists and it should be encouraged on educational, publication, and funding levels (Pankevich et al., 2011).

Future studies across all neuroscience disciplines and topics including PFC-mediated functions would benefit from the inclusion of both sexes. At the clinical level, understanding what makes one sex more protected from a disease could lead to the

development of more effective treatments and clear advantages exist at the experimental level as well. McCarthy et al. (2012) argued that among other reasons, studying sex differences in the past has led to fundamental discoveries such as adult neurogenesis, hormonal modulation of programmed cell death, neurosteroidogenesis, and prostaglandin-mediated synaptogenesis. Ultimately, clearer insight into the differences between males and females will benefit both sexes in the long-term.

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