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Hormone Influence on the Mesocortical Dopaminergic System: An Integrated Behavioral, Anatomical and *In Vivo* Physiological Characterization in Adult Male Rats

A dissertation presented by

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by

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In

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The prefrontal cortices (PFC) are linked to highest-order executive functions such as working memory, planning and decision making. These complex constructs are strongly dependent on local signaling via the neurotransmitter dopamine (DA). Stimulated by male over female sex differences in PFC function and especially by the significantly greater vulnerability of males to symptoms of PFC dysfunction in disorders such as schizophrenia, the experiments of this dissertation explored behavioral and biochemical effects of gonadal hormones on PFC DA systems using adult male rats that were gonadectomized (GDX) and supplemented with either androgens or estrogens as animal models. The first studies extended previous evidence that GDX disrupts performance in DA-dependent PFC behavioral tasks to the novel object recognition paradigm, which enabled the first identification of GDX effects on working memory independent of any effects on the animals' motivational state. Next, using *in vivo* microdialysis, it was found that resting PFC DA levels were nearly two-fold higher than normal in GDX animals. In searching for anatomical substrates for these androgen-sensitive, estrogen-insensitive effects of GDX, it was considered that fewer than 25% of the DA neurons that innervate the PFC contained androgen receptors, i.e. were androgen sensitive. Thus, the next study combined anatomical track-tracing and immunohistochemistry for androgen receptors to see whether neurons projecting to these DA cells might be the locus of hormone sensitivity. These studies revealed the surprising result that perhaps the most likely targets of androgen actions were neurons in the PFC itself that use glutamate as neurotransmitter. Thus, the final experiment again used *in*

vivo microdialysis, but this time paired with glutamate receptor-selective drugs. These studies revealed that GDX caused striking androgen-sensitive deviations from expected drug-stimulated changes in PFC DA levels. Taken together, these findings thus extend understanding of androgen's regulation of PFC function in males and identify DA/glutamate interactions as prime targets for this gonadal hormone influence. Because DA/glutamate interactions are central to contemporary etiological theories for disorders including schizophrenia, autism and ADHD, these findings may not only bring us closer to understanding the neurobiological bases for these devastating disorders, but closer to overcoming them as well.

Dedication

The past five years of my life spent as a graduate student in the Program in Neuroscience have taken me through some of the greatest highs and lows of my life. I have experienced both a personal and professional growth through the experience of learning my way as a scientist, forming my own ideas, going through the trials and tribulations of validating them, and coming away with an unshakeable foundation in knowing how to think, reason and evaluate. For this newfound confidence with which I can go out into the world as a better scientist and also as a better person, I would like to thank all those whose time, effort, and energy has contributed not only to this dissertation but also to my education and growth.

To my advisor, Dr. Mary Kritzer, I believe words are not enough to express the depth of my gratitude and appreciation, not only for her innumerable contributions to my development as a scientist but also for her care for me as an individual making her way into the world. Her intelligence, warmth, humor, personal care and attention, and above all her uncanny ability to bring out the best in those around her provided me with the best possible experience I ever could have asked for in a mentor and friend. I hope to continue a long-lasting personal and professional relationship with her, and I will always hold her in the highest regard. As well, members of the Kritzer laboratory, both past and present, have my unending gratitude for long days and nights of help and support along the way. I would also like to thank the members of my thesis committee, Drs. Craig Evinger, Lonnie Wollmuth, and

John Robinson for their thoughtful guidance and critique of the work presented herein. As well, I would like to recognize and thank Diane Godden, our departmental Graduate Program Coordinator, for going above and beyond the call of duty with all of her help throughout the entire process of graduate school and all that it requires.

Finally, though I will take many fond memories away from this experience, it would never have begun without the unwavering support and love of my family and loved ones. Their unshakeable confidence in me and their belief that I could do anything that I set my mind to made each and every day possible, and it is to them – my parents, Fred and Nona, my sister and biggest fan Cindy, my boyfriend Michael, my grandparents, aunts, uncles, cousins, and friends near and far – that I dedicate this dissertation. Thank you from the bottom of my heart.

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General Introduction

One of the major goals of neurobiology is to understand the biological bases of behaviors, from the most basic to the most complex. Until recently, different methods of addressing these questions had often focused on models that sought to characterize simple systems or sensory processes rather than more complex, higher-order processes mediated by areas such as the prefrontal cortex (PFC, Goldman-Rakic et al., 1990). Somewhat in parallel, studies of the contributions of gonadal steroid hormones in brain systems have only in more recent times expanded from focusing on their roles as stimulators of neuroendocrine function to regulators of not only complex behavior (e.g. Galea et al. 2008, Daniel, 2006, Gibbs, 2005) but perhaps as being involved in cognitive dysfunction in disease. For instance, sex differences and/or sensitivity to gonadal steroid hormones are not only important in the development and adult function of the PFC in humans, but have also been identified in psychiatric disorders such as schizophrenia and attention deficit hyperactive disorder (e.g. Xiang et al. 2010, Rucklidge, 2008, Nasser et al. 2002). Critically to the context of this dissertation, these disorders have also been linked to disturbances in the brain's dopamine (DA) systems (Viggiano et al., 2003; Hains and Arnsten, 2008; Oades, 2008; Arnsten, 2009; Del Arco and Mora, 2009; Dremencov et al., 2009; Scarr and Dean, 2009). Accordingly, there has been a growing interest in using animal models to discern the role(s) of gonadal steroid hormones in modulating DA. The studies in this dissertation add to this growing body of literature by exploring how gonadal steroid hormones, particularly androgen,

affect an array of behavioral, anatomical, and physiological endpoints associated with the mesocortical DA system in adult male rats. The introductory sections below will first explain the importance of the PFC and its DA innervation and outline previous studies regarding its hormone and especially androgen regulation in both humans and in animal models. Then, the research goals of this dissertation will be framed, and finally a rationale for the progression of the studies contained herein will be presented.

The Prefrontal Cortex and its Dopamine System

In humans and animals alike, the prefrontal cortices are a series of forebrain regions - medial, orbital, and perirhinal - linked to executive, mnemonic, affective and other higher-order functions such as planning, working memory, and behavioral flexibility (see Goldman-Rakic 198; Dalley 2004). These and other PFC-mediated functions are disrupted in diseases such as schizophrenia and attention deficit hyperactivity disorder in humans and have been correlated with hypofrontality (Weinberger et al., 1986; Zametkin et al., 1990). Interestingly, in humans and animal models alike, these same functions are critically dependent upon the proper regulation of the PFC DA system, which arises from the midbrain ventral tegmental area (VTA, see Figure In.1). For instance, in humans PFC dysfunctions not only in disease but as a result of normal aging are all linked to diminished prefrontal DA (Weinberger et al, 1986, 2001; Akil et al., 1999; Mattay et al., 2002). In animals, the essential input of DA to PFC function has been carefully demonstrated under a variety of experimental conditions. For example, in rats, chemical lesions of PFC DA afferents (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989;

Stam et al., 1989), local administration of dopamine D1 receptor agonists and antagonists (Zahrt et al., 1997; Winter et al., 2009) as well as β-carboline- and stress-induced increases in DA turnover (Murphy et al., 1996; Morrow et al., 2000; Moghaddam and Jackson, 2004) all adversely affect performance in frontal lobe-dependent tasks including open field testing, delayed alternation paradigms and novel object recognition. These reports illustrate a point fundamental to the studies contained in this dissertation -- that PFC function depends on the proper regulation of PFC DA tone, and that that both too much and too little DA in the PFC is therefore detrimental to normal PFC function.

Hormone Regulation of Prefrontal Cortical DA: Schizophrenia

Sex differences and/or gonadal steroid hormone regulation has been identified in many human disorders ranging from Parkinson's disease to drug addiction to schizophrenia (see Di Paolo 1994; Nasser et al. 2002) that are further linked to brain DA systems. This section will focus on schizophrenia as a representative disease, both due to its sexual dimorphisms in incidence and pathology (e.g. Xiang et al. 2010; Nasser et al. 2002) and its specific disruptions in mesocortical DA systems. Schizophrenia is a mental disorder characterized by abnormalities in the perception or expression of reality. It is associated with specific disruptions in the mesolimbic and mesocortical DA systems in humans. Specifically in male patients, studies have identified abnormalities in circulating testicular hormones (Mason et al., 1988) and a prevalence of negative symptoms (see Seeman and Lang, 1990). Most compellingly, studies across numerous independent patient populations have significantly correlated decreased (25-50%) testosterone levels with the severity of the negative,

PFC symptoms of this devastating disorder, which are its most difficult to treat (Ko et al., 2007; Akhondzadeh et al., 2006, Huber et al., 2005; Goyal et al., 2004; Taherianfard and Shariaty, 2004; Shirayama et al., 2002). That these symptoms specifically involve PFC DA systems and occur disproportionately in males (Xiang et al. 2010) make this both an area of basic scientific and clinical interest, as a better understanding of the mechanisms of hormone stimulation involved could lead to improved clinical outcomes. Of special interest to this dissertation, in contrast to the large number of studies and great strides that have been made in identifying roles for gonadal steroids in cognitive functions in females (see Gibbs and Gabor, 2003), corresponding experimental studies in males are very few.

Animal Studies of Hormone Regulation of Prefrontal Cortical DA: Androgen Regulation in Adult Male Rats

Studies in rats and mice provide strong hints about gonadal hormone and especially androgren contributions to PFC DA systems in males. While there is some evidence that hormone influence over the development of mesocortical DA and other cortical catecholamine systems occurs in rats (e.g., Battaner et al., 1987; Stewart et al., 1991), contributions of hormones in adult animals have also been seen. For example, male rats show superiority to females in certain spatial learning tasks (Dawson et al., 1975) and in acquiring (Van Hest et al., 1988) and performing working memory tasks (Einon, 1980; Tees et al, 1981). In studies testing DA systems specifically within adult male rats, gonadal hormones are removed via surgical methods (gonadectomy, GDX) and estrogen, testosterone or the non-aromatizeable androgen dihydrotestosterone are replaced in cohorts of

gonadectomized animals to isolate effects of androgrens, estrogrens, or synergistic effects of the two. In the GDX adult male rat model used here, plasma testosterone levels are typically reduced by 75-90% (Adler et al., 1999; Turvin et al., 2007). While these decreases are typically more severe than those seen in schizophrenic men, this model is still applicable in identifying neural substrates that are sensitive to changes in the hormonal milieu, characterizing the nature of their dysregulation in the GDX groups, and addressing androgen- or estrogen-sensitive regulation of PFC DA systems in males.

The impact of androgen specifically on PFC DA systems and behaviors in adult male animals has been previously demonstrated in anatomical observations, which initially showed that long-term GDX in adult male rats significantly increases the density of axons immunoreactive for the DA synthesizing enzyme tyrosine hydroxylase (TH) in the anterior cingulate cortex --part of the medial prefrontal cortex -- but not primary somatosensory or motor cortex (Kritzer et al., 1999). That observation has been followed up by studies demonstrating that effects on TH reflect changes specifically in DA afferents and not norepinephrine afferents (Kritzer, 2000; Kritzer 2003), that TH axon density is increased in all major PFC cortical fields (Kritzer, 2003), and critically that that these effects are all attenuated by replacing rats with the non-aromatizable dihydrotestosterone but not estrogen (Kritzer, 2000). While these anatomical findings were interesting and certainly encouraging, what makes the GDX model of critical importance to this dissertation were findings of similarly androgen-sensitive, estrogen-insensitive effects of GDX on DA-dependent PFC functions. For example, open field testing, which is a paradigm sensitive to

surgical ablations of PFC (Holson and Walker, 1986), to chemical lesions of its DA afferents (Kalsbeek et al., 1989; Tassin et al., 1978) is also sensitive and to GDX in adult male rats (Adler et al., 1999). Likewise, acquisition of T-maze delayed alternation, a spatial working memory test that is sensitive to PFC DA manipulations such as chemical lesions (e.g., Kessler and Markowitsch, 1981; Stam et al., 1989), receptor stimulation (Zahrt et al., 1997) and induced increases in prefrontal DA turnover (Verma and Moghaddam, 1996; Murphy et al., 1997) is also impaired by GDX in an androgen-reversible way (Kritzer et al., 2001). Perhaps most intriguingly, more recent behavioral studies have correlated the increase in TH axon density that occurs in GDX animals with impaired individual performance in DA-dependent tasks in both the orbital and medial subdivisions of the PFC within the same animals (Kritzer et al. 2007). Thus, the convergence of anatomical and functional findings in GDX rats suggests that this model system may allow for the ultimate goal of mapping biology onto cognitive behavior.

Modulation of PFC Systems by Dopamine: Extracellular Levels and Physiology

While these previous studies have taken steps to correlate GDX effects with PFC DA, the fact remains that they do not directly measure changes in PFC DA in this animal model; that is, they do not show that circulating androgens can influence PFC function by selectively influencing DA levels or physiology within these cortical fields. Under normal, basal conditions, the PFC-projecting DA neurons from the VTA in rats exhibit high rates of single-spiking (tonic) activity (see Overton and Clark, 1997; Grace and Onn, 1989). In response to behaviorally relevant or otherwise salient stimuli the firing mode of these neurons shifts to a characteristic multi-spike bursting

pattern (Freeman and Bunney, 1987; see Overton and Clark, 1997; Hyland et al., 2002). While the changes in firing rate of the neurons in the VTA may occur dynamically, in the PFC this activity is translated into phasic changes in extracellular DA levels that occur much more gradually and can last for up to tens of minutes after short epochs of DA neuron stimulation or VTA bursting activity (Garris et al., 1993; Cass and Gerhardt, 1994). Thus, while the activity of VTA DAergic neurons may encode temporal information about behavioral contingencies or attentional processes (Freeman and Bunney, 1987, see also Schultz, 1997, 1998), information from microdialysis (e.g., Watanabe et al., 1997; Finlay and Zigmond, 1997; Feenstra et al., 2000), voltammetry and electrophysiology (e.g., Lavin et al., 2005) and computational models (Compte et al., 2000; Durstewitz et al., 2000; Durstewitz and Seamans, 2002) suggests that DA in the PFC may serve to bias local network properties in PFC circuits in relation to information gathered over a long period of time. This modulatory influence is specific and sophisticated in the PFC and involves the actions of functionally disparate DA receptor subtypes and specific subsets of sets of inhibitory and excitatory cortical neurons. However, the end result seems to indicate that DA acts as neither as an overall excitatory nor inhibitory influence, but rather works as a modulator; it has the ability to simultaneously suppress activity in some cells and augment it in others (see Robbins and Arnsten, 2009; O'Donnell, 2004). Thus, it is in the position to control the complex tuning properties that are required of PFC neuronal networks in orchestrating highest order information processing (Durstewitz et al., 2000; Durstewitz and Seamans, 2002; Seamans et al., 2001). Thus, the singular nature of extracellular DA levels in the PFC make this

basic measure of pivotal importance to PFC function and perhaps its dysfunction in disease - if extracellular PFC DA levels are disrupted in GDX animal models, it may indicate a dysregulation of the local PFC network. Therefore, one of the primary goals of this dissertation was to measure extracellular PFC DA levels in the GDX animal model and to investigate testable hypotheses regarding hormone regulation of this parameter.

Questions Addressed in this Dissertation

While there could be many endpoints by which hormones might contribute to setting and maintaining extracellular prefrontal DA levels, the initial studies in this dissertation sought to expand evidence of androgen influence over DA-dependent PFC function to the final subdivision of this region - the perirhinal cortex - and then to measure extracellular DA levels in the medial PFC of sham-operated, GDX, and GDX animals replaced with either testosterone or estrogen in order to find out if anatomical changes seen in this animal model translate into physiological changes in the pivotal measure of extracellular DA. These studies are presented in Chapters I and II of this dissertation, where GDX was seen to affect a specifically perirhinal prefrontal cortical DA-dependent task, Novel Object Recognition, in an androgen-sensitive, estrogen-insensitive manner. Studies in this same animal model using *in vivo* microdialysis also revealed that GDX animals have a nearly two-fold increase in extracellular DA in the medial PFC, and that this increase is likewise reversed by replacement with androgen, but not estrogen. Since the major DAergic pathway to the PFC arises from the VTA, the immediate hypothesis raised by these findings was that the DAergic cell bodies in the VTA would themselves be androgen-

sensitive. However, retrograde tract-tracing from injections into the PFC combined with double-label immunocytochemistry for nuclear androgen receptors has previously shown that most of the PFC-projecting DAergic cell bodies from the VTA do not themselves contain AR (Kritzer and Creutz, 2008). Thus, the third chapter of this dissertation sought to characterize other possible loci of hormone influence over the DAergic neurons in the VTA by likewise utilizing retrograde tract-tracing from injections into the VTA combined with double-label immunocytochemistry for AR to characterize the possible androgen sensitivity of all projections to the ventral midbrain. Having found, rather surprisingly, that over half of the glutamatergic projections from the PFC itself to the VTA are immunoreactive for androgen receptor and thus likely to be candidates for hormone stimulation, the final chapter of this dissertation (Chapter IV) again used *in vivo* microdialysis paired with glutamate receptor subtype-specific antagonists in order to discern the contributions, if any, of androgen to glutamate-stimulated PFC DA levels. In accordance with the interest here in not only elucidating neurobiological mechanisms of hormone stimulation but in improved clinical outcomes for diseases such as schizophrenia, impetus for further exploring the newly found hormonal influence over PFC glutamate/dopamine interactions seen in Chapter IV of this dissertation comes from evidence that both of these neurotransmitters are also at the heart of current etiological theories of schizophrenia, as it has been posited that the negative symptoms of this disorder may be explained by primary defects in the PFC glutamatergic system, with secondary effects on PFC DA tone (Coyle et al., 2003; Moghaddam, 2003; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010).

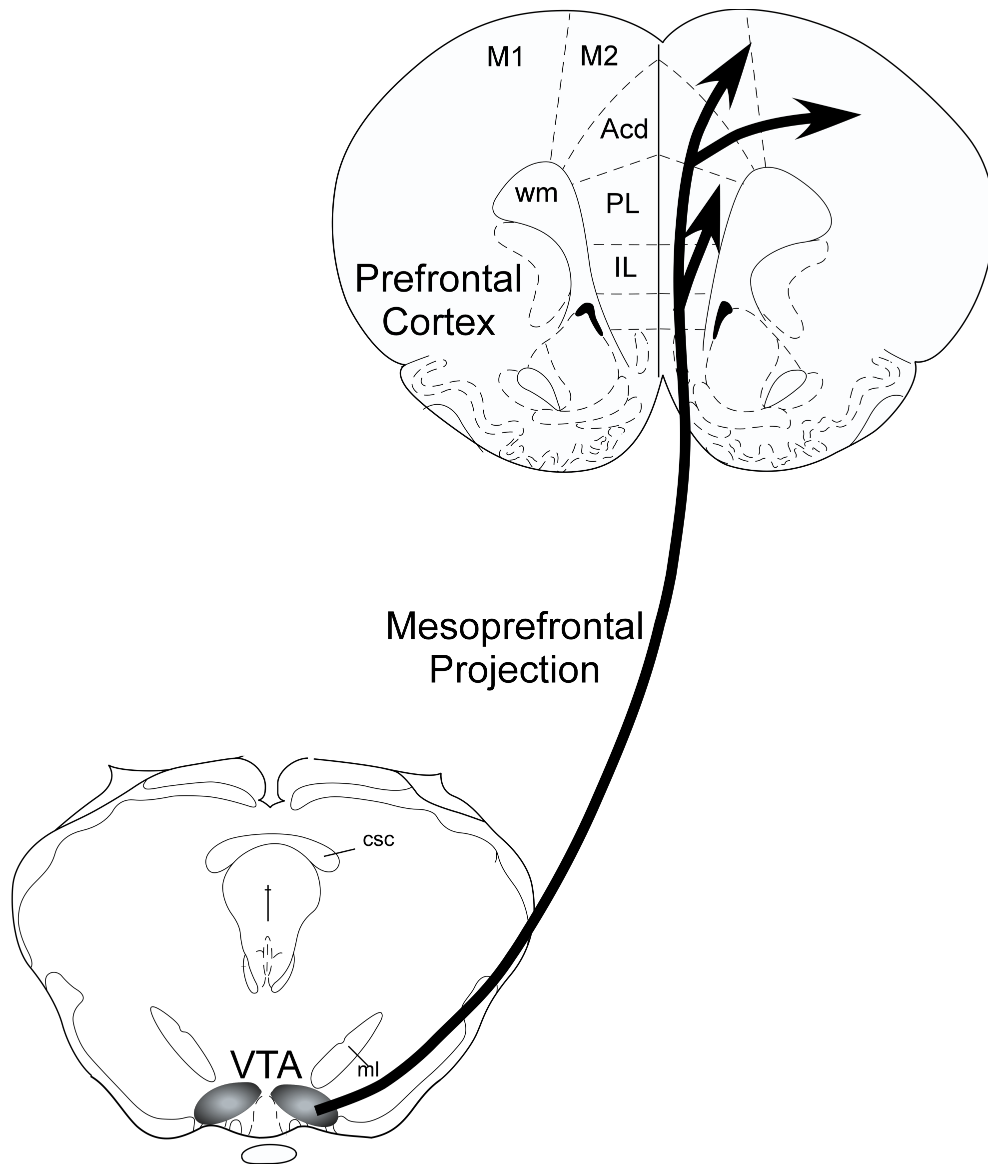

Figure In.1. A schematic representation of the major dopaminergic projection from the ventral tegmental area (VTA) to the prefrontal cortex in the adult male rat model. Section outlines are adapted from the atlas of Paxinos and Watson (1998). Additional abbreviations: csc, commissure of superior colliculus; ml, medial lemniscus; IL, infralimbic cortex; PL, prelimbic cortex; Acd, anterior cingulate cortex; M2, premotor cortex; M1, motor cortex; wm, white matter

Chapter I

Effects of gonadectomy and hormone replacement on a spontaneous novel object recognition task in adult male rats

Although there may be a longer history of study regarding gonadal hormone influence over the cognitive, mnemonic and executive functions of the prefrontal cortices in females (Berman et al., 1997; Gibbs and Gabor, 2003; Keenan et al., 2001; Korol, 2004), there is growing evidence that gonadal steroids also impact prefrontal cortical operations in males. For example, in both young and aged men, circulating testosterone levels have been positively correlated with performance in prefrontal tasks including mental rotation, verbal recall and divided attention (Cherrier et al., 2002; Cherrier et al., 2001; Christiansen and Knussmann, 1987; Moffat and Hampson, 1996; Yaffe et al., 2002). Studies in animal models also suggest an importance of gonadal steroid stimulation for prefrontal cortical function in males. For example, gonadectomy in adult male rats has been shown to significantly impair performance in maze and operant tests of spatial (Daniel et al., 2003; Kritzer et al., 2007; Kritzer et al., 2001) and non-spatial (Ceccarelli et al., 2001) working memory and behavioral flexibility (Kritzer et al., 2007), which are processes that are dependent on the medial prefrontal cortices (Dias and Aggleton, 2000; Kesner et al., 1996; Lacroix et al., 2002; Schwabe et al., 2004; Taylor et al., 2003), as well as performance on a progressive reward ratio task (Kritzer et al., 2007) which is sensitive to lesions of the orbital prefrontal cortices

(Kheramin et al., 2005). Thus, in this chapter , analyses of hormone sensitivity were extended to a task sensitive in part to lesions of the perirhinal prefrontal cortices, a spontaneous novel object recognition (NOR) task, to test the hypothesis that functions mediated by this third major division of the prefrontal cortex may also be sensitive to long-term gonadectomy and hormone replacement in adult male subjects. The novel object recognition task is in part a working memory paradigm that is sensitive to both hippocampal (Gaskin et al., 2003; Gould et al., 2002; Gulinello et al., 2006) and cortical lesions placed in and around the prefrontal areas surrounding the rhinal fissure (Aggleton et al., 1997; Barker et al., 2007; Buffalo et al., 2006; Cowell et al., 2006; Ennaceur et al., 1996; Ennaceur et al., 1997; Moses et al., 2005; Mumby and Pinel, 1994; Winters et al., 2004). It is also a task where hormone sensitivity has been previously established in findings of sex and/or estrous cycle differences in NOR performance (Bisagno et al., 2003; Ghi et al., 1999; Sutcliffe et al., 2007; Walf et al., 2006), and in the attenuation of NOR deficits in ovariectomized female rats (with and without chronic stress) by giving ovariectomized animals estradiol (Luine et al., 2006; Luine et al., 2003; Wallace et al., 2006). Here, NOR performance was assessed for the first time in gonadectomized and hormone-replaced adult male rats. In view of previous evidence for effects of gonadectomy on open field behavior (Adler et al., 1999; Kerr et al., 1996; Slob et al., 1986) quantitative assessments of all major behaviors exhibited by the animals

including ambulation and rearing were also made alongside those of object exploration to determine whether and to what extent hormone effects on these ancillary behaviors might affect outcome measures of novel object recognition and/or discrimination in males.

Methods

Animal Subjects: Thirty-one adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Rats weighed 200-250g at time of surgery and 275-400g when testing began. Animals were divided into four treatment groups: sham operated controls (*n*=8, CTRL), rats that were gonadectomized (*n*=8, GDX), and gonadectomized rats that were supplemented with testosterone propionate (*n*=7, GDX-TP), or 17-β-estradiol (*n*=8, GDX-E). Throughout, subjects were housed under a 12/12 light/dark cycle with food and water available *ad libitum* in home cages that contained 2-3 animals; individuals from each treatment group were housed with a mix of individuals either from other or their own treatment groups. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize their stress and discomfort.

Surgical Procedures and Hormone Replacement: Sham and GDX surgeries were performed 21 days before behavioral testing began under aseptic conditions and using intraperitoneal injections of ketamine (2 mg/100g) and xylazine (1 mg/100g) for anesthesia. For all surgeries, the sac of the scrotum and the underlying layers of tunica were incised. For

GDX, the vas deferens was then ligated bilaterally and the testes removed. For the hormone-replaced groups, slow-release pellets that release approximately 3–4 ng of TP per milliliter of blood per day or 25 pg of E per milliliter of blood per day (Innovative Research of America, Sarasota, FL) were then inserted within the tunica. These pellets have been used in previous investigations in this laboratory and have been shown to produce circulating levels of gonadal hormones in GDX rats that fall within physiological ranges (Adler et al., 1999; Kritzer et al., 2007). Incisions were closed using surgical staples.

Euthanasia*:* Animals were euthanized two days after completion of behavioral testing by rapid decapitation and their brains were removed and frozen for use in unrelated receptor binding studies. The medial, ventral, and lateral bulbospongiosus muscles (BSM) were also dissected out and weighed at this time.

Behavioral Testing: Apparatus and General Procedures:

Apparatus: Testing was conducted in an open-field arena (80x30x50 cm) that had walls and a floor made of opaque plastic. A grid of 24 10x10 cm squares was marked on the arena floor, and a digital video camera was suspended overhead to record behavior during all testing sessions. A 70% ethanol solution was used to clean the testing arena and all objects (below) before and between all individual trials on all testing days. All tests

were conducted in a dimly-lit room with a low level of background white noise (50 dB).

Objects: Common, duplicate objects of varying shapes and sizes that included coffee mugs, small and large drinking glasses, teacups, soda cans, and bottles were used for testing object bias and object recognition. All objects weighed at least 450g to discourage their being moved by the animals during testing. The smallest object was $5cm^2$, and the largest had dimensions of roughly 24cm x 12cm. For object recognition, all objects were approximately cylindrical in shape and ranged from 20-24cm in height and 8-12cm in width.

Timeline: Testing took place over a six day period during the animals' subjective night, between 0800-1500 hours, when the lights in their home environment were on. Prior to testing, the animals' home cages were placed in the testing room for a period of one hour. The object bias test was conducted first, followed on the next day by an open field test, and on the day after that with an object recognition task habituation trial. After a two day rest interval, the object recognition trials began, with tests using a 1.5 and 4h delay performed sequentially separated by one additional, intervening day of rest. The two delay periods were chosen to model previous studies of hormone manipulations in adult female animals (Luine et al., 2006; Luine et al., 2003; Walf et al., 2006).

Behavioral Testing: Paradigm-specific Procedures:

Object Bias Testing: Animals were placed in the open-field arena with four objects belonging to four categories that were differentiated by size and shape/geometric complexity. Criteria for the categories were: large (more than to 18cm tall), small (less than 12cm tall), smooth (having a regular, cylindrical shape), and complex (having sharp angles, curves, or extending features), and the object categories were: small/smooth objects (e.g., small bowl); large/smooth objects (e.g., soda can); small/complex objects (e.g., teacup) and large/complex objects (e.g., coffee mug). All were located equidistantly from each other and from the arena corners during testing. Animals were placed at the center of the open field to start, with the starting direction and corner positions of the objects falling into the four categories counterbalanced within and across groups.

Open Field Test: Animals were placed in the center of the empty arena and allowed a six-minute period to explore. Observation revealed that the principal behaviors engaged in could be categorized as ambulating, rearing or remaining stationary. Times spent on these three behaviors were measured separately for the first and second halves of the six minute testing period.

Object Recognition Task Habituation: Identical pairs of objects were placed at one end of the arena 10 cm away from all adjacent walls. Animals were started at the opposite end of the arena facing away from the objects and given three minutes of exploration time. The times spent ambulating, stationary, rearing, and actively exploring objects was scored separately for the first and second halves of the testing period.

Object Recognition: The object recognition task consisted of an initial 3-minute sample trial and a subsequent 3-minute test trial. During the sample trial, an identical pair of novel objects (not used in any previous testing) was placed at one end of the arena and in the test trial, one of the pair of sample objects was replaced by a novel object. For all trials, animals were started in the opposite end of the arena facing away from the objects, and the objects used and the positions of the novel and familiar objects during the testing phase were counterbalanced within and across animal groups. All animals were tested first at a 1.5h trial delay and two days later at a 4 hour delay. All behaviors including object exploration and the discrimination index (DI, below) were scored separately for the first and second half of the sample and test trials.

Analysis:

Behavioral Definitions: Behaviors were quantified from digital recordings by a single, blind observer. “Ambulation” was defined as the crossing of at

least 1 floor grid line within a 3 second period, “stationary” corresponded to the animal remaining unmoving for at least a 3 second duration, and “rearing” was defined as a lifting of the forelimbs and sitting back upon the haunches. Exploration of objects was defined as the animal directing its nose to an item and actively sniffing or whisking; facing or climbing on the object alone did not meet this criteria.

Discrimination Index: The differential exploration of two items was quantified by calculating a discrimination index (DI), in which the difference in the amount of time spent with one object vs. the other is expressed as a proportion of total exploration time. When both objects were identical, this was computed according to the following formula, where Obj 1 signified the object on the left, and Obj2 signified the object on the right of the apparatus:

$$(\frac{Obj1Time - Obj2Time}{Obj1Time + Obj2Time})$$

During novel object testing, the DI was calculated as*:*

$$(\frac{NovelTime - FamiliarTime}{NovelTime + FamiliarTime})$$

With this analysis, a positive score indicated more time spent with the novel object, and a negative score indicated more time spent with the familiar object. Data were collected in separate epochs for the first and second halves of the testing sessions.

Statistics: Times spent on given behaviors were expressed as percents of total (object bias testing) or half (all other testing) trial times for statistical analysis and comparison. Assessments of descriptive statistics (mean, variance) were followed by allowed comparisons using single or repeated-measures ANOVAs that included separate measures of all behaviors (StatView 5.0). The DI and bulbospongiosus muscle weights were also compared using regression analyses. For all statistical assessments, post-hoc testing used the Student-Neuman-Keuls (SNK) test, a $p<0.05$ level was accepted as significant, and a $p<0.09$ level was identified as the cut-off for group differences that approached significance.

Results

Effectiveness of Hormone Treatment:

Bulbospongiosus muscle (BSM) weights showed expected group differences. Thus, the CTRL and GDX-TP animals had average BSM weights of 1.22 and 1.17g, respectively, whereas GDX and GDX-E animals had average BSM weights of 0.30 and 0.27g, respectively. An ANOVA that compared weights across treatment groups revealed significant main effects of hormone treatment [$F_{(3,27)}=59.01$, $p<0.0001$], and post hoc tests confirmed that the androgen-sensitive BSM weights of the control and GDX-TP groups did not differ from one another, that the weights in GDX and GDX-E animals were not significantly different from

each other, but that the BSM weights of both the GDX and GDX-E groups were significantly lower than those of both the control and GDX-TP groups (Figure I.1).

Object Bias Test:

Testing for object bias revealed consistent differences in the amounts of time that the animals spent exploring four concurrently presented objects belonging to four different size/shape categories, but no group differences in these biases. Thus, the sham-operated, GDX, and GDX-TP and GDX-E cohorts alike all spent roughly 35-38% of total exploration time focusing on large, complex objects, 22-35% of time investigating smaller contoured objects, 20-24% of time on large, smooth objects, and the least amount of time (~14-19%) exploring the smallest, smoothest objects present (Figure I.2). A one-way ANOVA that compared proportionate exploration times confirmed that there were significant main effects of object type/category [$F_{(3,\ 31)}$=14.51, p< .0001] but no significant main effects of hormone treatment [$F_{(3,\ 31)}$=.440, p=.7263] or hormone treatment by object category interactions [$F_{(3,\ 31)}$=1.474, p= .1715] on exploration times. Post-hoc testing also confirmed that most time was spent exploring large, complex objects (p=.0222-.0365), and significantly to near-significantly less time was spent interacting with small, smooth ones (p=.0301-.0593) (Figure I.2 A, D). Based on these results, all subsequent testing that required objects used those falling under the classification of large/smooth; objects in this category elicited robust

exploration from all animal groups and their relatively simple geometries allowed for a high degree of form continuity among the like and unlike objects used in testing.

Open Field Test:

During Open Field testing, all animal groups engaged in the same three principal behaviors (ambulation, rearing and adopting stationary postures), and all groups apportioned their time among these activities similarly. Thus, during the first half of the trial, animals spent 57-70% ambulating, 20-33% of time stationary and about 6-10% of time rearing. For all groups as well, the percentage of time devoted to ambulating decreased by about 30%, the time spent stationary increased by a similar degree, and the time spent rearing did not appreciably change from the first to the second half of the testing period (Figure I.3). An ANOVA with repeated-measures design that included separate values for time spent on the three principal activities during the first and second halves of the testing period confirmed that there were significant main effects of behavior type [$F_{(2,3)}=25.461$, $p<.0001$], and significant main effects of testing half [$F_{(2,3)}=71.874$, $p<.0001$], but that there were there were no significant main effects of hormone treatment [$F_{(3,25)}=.774$, $p=.5192$] or significant interactions between hormone treatment and behavior type [$F_{(3,6)}=.361$, $p=.9003$] or testing halves [$F_{(3,6)}=.789$, $p=.5830$, see Figure I.3] on open-field activities.

Object Recognition Task Habituation:

Statistical analyses (ANOVA with a repeated-measures design) of a habituation trial for the spontaneous novel object recognition task that included measures of time spent ambulating, rearing, stationary and actively exploring the pair of identical objects that were present in the testing arena revealed significant differences in the amounts of time spent on specific behaviors [$F_{(3, 25)}$=55.672, p<.0001], and in the proportions of time allotted to them during the first and second halves of testing [$F_{(3, 1)}$=53.402, p<.0001, see Figure I.4A], but no significant main effects of hormone treatment [$F_{(3, 25)}$=1.504, p=.3863] or significant interactions between hormone treatment and specific behaviors [$F_{(3, 25)}$=.291, p=.9752] or activities. There were also no significant effects of hormone treatment [$F_{(3, 25)}$=1.5, p=.3661] or significant effects of hormone treatment on the computed discrimination index (DI) of object exploration [$F_{(3, 25)}$=.281, p=.8252], whose consistently near zero values indicated that all animals and animal groups tended to interact with each of the two identical objects present for similar amounts of time (Figure I.4B).

Novel Object Recognition Tasks (1.5 h and 4 h delay):

During spontaneous novel object recognition testing two CTRL, one GDX and one GDX-TP animal moved from the start position immediately to a corner of the testing box and remained there for the entire sample

trial; data from these four animals were thus excluded from the analyses below.

As observed in the Open Field and Object Recognition Task Habituation tests, significant differences were identified in the percentages of time that the animals spent ambulating, rearing and stationary during the first halves of testing and in the redistributions of time spent on these activities during second halves of the three-minute periods for both the 1.5 (all $p<.0001$, see Figure I.5 A, B) and the 4 hour delay tasks ($p<.0004$-.0001 Figure I.6, A, B). For the most part, there were no significant group differences in these measures. However, three subjects from the GDX-TP group did show unusually pronounced decreases in ambulation and rearing during the second half of testing for the 1.5 hour delay sample trial, and the repeated-measures ANOVA that included these data uniquely revealed interactions between hormone treatment and behavior [$F_{(3,9)}=3.817$, $p<.0005$] and between hormone treatment and behavior over testing halves [$F_{(3,9)}=2.640$, $p<.0129$] that were significant. Post-hoc testing, however, confirmed that these effects were driven largely by the ambulatory and stationary activities of the outliers from the GDX-TP group (Figure I.5 B).

During both the sample and test trials for the two NOR tasks evaluated (1.5 and 4 h delay) all animal groups engaged in similar absolute and proportional amounts of object exploration time (Figures I.5 A, B; I.6 A, B). The DIs of object exploration measured during the sample

trials also showed that all groups explored the two identical objects present equally (Figures I.5C, 6C). Statistical analyses of the sample trial (ANOVAs) confirmed that there were no significant main effects of hormone treatment [1.5 hour delay: $F_{(3,\ 25)}$=.327, p=.8061; 4 hour delay: $F_{(3,\ 25)}$=.319, p=.8118] or testing halves on the DI [1.5 hour delay: $F_{(3,\ 1)}$=.012, p=.9143; 4 hour delay: $F_{(3,\ 1)}$=.559, p=.4618], nor significant interactions between hormone treatment and DI [1.5 hour delay: $F_{(3,\ 1)}$=.607, p=.6172; 4 hour delay; $F_{(3,\ 1)}$=.325, p<.8073].

In the test trials, the CTRL and GDX-TP groups spent proportionately more time exploring the novel compared to the familiar object present, whereas GDX and GDX-E groups continued to divide exploration time equally between the old and new objects (Figures I.5D; I.6D). This group difference was especially pronounced during the first half of the test trials and less so during the second half, during which time the GDX and GDX-E animals persisted in splitting their time evenly between objects and the CTRL and GDX-TP groups attended to the novel and familiar objects more and more similarly. Analyses of variance with repeated-measures designs confirmed these observations and revealed that for the 1.5 hour delay there was a significant main effect of hormone treatment on DI during the first half [$F_{(3,24)}$=6.034, p< .0033, Figure I.5D] but not the second half of the test trial. Allowed post-hoc testing further showed that while the DI's of GDX and GDX-E groups on the one hand and of the CTRL and GDX-TP groups on the other did not differ from each

other, the DI's of the GDX and GDX-E cohorts were both significantly different from those of the CTRL and GDX-TP groups for the first but not the second half of testing. Corresponding analyses for the 4 hour delay study revealed similar trends in the data that approached but did not reach significance (Figure I.6D). Finally, regression analyses that compared individual animal DI values with measures of their BSM weights (Figures I.5 E, F; I.6 E, F) likewise also showed that there was a significant correlation between high BSM weight and a high DI value (i.e., novel object preference) for the test (R^2=.289, p< .0032, Figure I.5F) but not the sample trial (R^2=.002, p<.8211, Figure I.5E) of the 1.5 hour study, and correlations between BSM and DI value that were stronger for the test than the sample trial but did not reach significance for the four hour study (Figure I.6 E, F).

Discussion

Recent studies in adult male rats have identified modulatory roles for gonadal steroids on working memory and other types of cognitive tasks that are known to be sensitive to lesions of the medial (Ceccarelli et al., 2001; Daniel et al., 2003; Kritzer et al., 2007; Turvin et al., 2007) and orbital (Kheramin et al., 2003; Kritzer et al., 2007) divisions of the prefrontal cortices in rats. The studies presented here now demonstrate gonadal hormone sensitivity for a spontaneous NOR task that is sensitive in part to lesions of the third major subdivision of the rat PFC – the

perirhinal prefrontal cortices. Specifically, long-term gonadectomy in adult male rats was found to significantly and selectively decrease the initial discrimination index in a spontaneous object recognition task at a 1.5 hour delay. Like previously observed effects of gonadectomy on other prefrontal cortical functions (Kritzer et al., 2007), the especially apparent deficits that marked the first half of testing of the 1.5 hour NOR task were attenuated by supplementing GDX animals with testosterone propionate but not estradiol and were significantly correlated with the somatic measure of circulating androgens, the weights of the bulbospongiosus muscle group (measured post-mortem within 2 days of the completion of behavioral testing). That hormone effects were greatest during the first half of test trials also aligns with previous data showing that in intact animals it is primarily during the initial part of the recognition phase of the test trials that a novel object is preferentially explored (Dix and Aggleton, 1999; Moses et al., 2005); after this initial exploration, novel objects rapidly become familiar, and control DI values decline, leaving in essence no room for deficits on novelty recognition to be resolved. Such basement effects may also explain why hormone effects seen in this study at the 4 hour delay were small and failed to reach significance. More specifically, given the relatively low DI of the CTRL group and the consequent reduction in the magnitude of between-group differences, the power needed to resolve group these differences statistically may have been raised beyond that possible with the experimental *n*'s of this study.

Behavioral Impact of GDX on the NOR Task.

Although there are several behavioral paradigms in rats that tap perirhinal prefrontal cortical functions, the focus here on gonadal hormones gave the selection of a NOR task several significant advantages. For example, given evidence for effects of gonadectomy and/or hormone replacement in adult male rats on measures of motivation including break point in a water- but not food-rewarded progressive reward ratio task (Kritzer et al., 2007; van Hest et al., 1988), acquisition of conditioned place preference, and in intracerebroventricular hormone self-administration (Wood, 2004), it is important that the NOR paradigm unlike other perirhinal tasks relies on spontaneous rather than rewarded activity. Further, because some of the effects of GDX on working memory paradigms include task acquisition (Kritzer et al., 2007), that the NOR task requires no training allowed hormone effects on mnemonic components of the task to be demonstrated in relative isolation from learning. Nonetheless, there were also balancing concerns about using the NOR paradigm. Most notably, as has been described in other studies like the present that used fixed trial times (Dix and Aggleton, 1999; Ennaceur and Delacour, 1988; Luine et al., 2003), outcome measures related to object exploration can be influenced by the amounts of time that subjects spend on other, essentially open field behaviors during testing (Moses et al., 2005). This was of particular concern here since significant differences in open field activity have been identified across the sexes (Blizard et al.,

1975; Slob et al., 1981; Slob et al., 1986; Swanson, 1966), between ovariectomized and hormonally replaced female rats (Frye and Walf, 2004; Heinsbroek et al., 1988; Palermo-Neto and Dorce, 1990), and among gonadectomized and hormone-replaced adult male rats (Adler et al. 1999; Swanson et al., 1966; but see Slob et al. 1981). The effects of GDX and hormone replacement in adult male rats on various measures of anxiety have also been identified in elevated plus maze (Frye and Walf, 2004; Walf and Frye, 2005a; Walf and Frye, 2005b), avoidance (Edinger and Frye, 2007; Frye et al., 2004) and acoustic startle response paradigms (Turvin et al., 2007), thus introducing the further possibility that anxiety in the GDX and/or hormone replaced subjects could affect NOR testing and outcome. For these reasons, detailed analyses of videotaped trials were included in the present studies. Importantly, these additional assessments failed to find any evidence for potentially confounding group differences along behavioral dimensions other than the DI of object exploration. Thus, none of the animals exhibited any obvious thigmotaxis or freezing behavior indicative of elevated anxiety, and quantitative analyses of the major behaviors that were observed in addition to object exploration—ambulation, rearing and remaining stationary—revealed no group differences in either the amounts of time devoted to these activities, or in the systematic changes in the proportions of time allotted to them that occurred as the trials progressed. Critically, there were also no significant group differences in the amounts of time that animals or animal

groups spent interacting with objects, which compared to the other behaviors evaluated was also surprisingly stable from the first to the second halves of testing for all animal groups. Taken together, these analyses indicate that it is unlikely that hormone effects on potentially interfering behaviors (e.g. ambulation) contributed to the group differences in the DI. Rather, whereas the central nervous system effects of gonadectomy and hormone replacement are widespread, behavioral effects of the manipulations on the spontaneous NOR task seem focused on the principal construct of this paradigm—novel object recognition.

Hormone Sensitivity of the NOR Task: Comparison to Previous Studies.

Previous studies have examined spontaneous novel object recognition across the sexes or in hormone-manipulated female rats or mice. In these, although there are discrepancies regarding effects of the estrous cycle on NOR performance (Sutcliffe et al., 2007; Walf et al., 2006), evidence of superior NOR in gonadally intact female compared to male rats (Ghi et al., 1999; Sutcliffe et al., 2007) and in gonadally intact and ovariectomized female rats supplemented with estradiol compared to ovariectomized controls (Luine et al., 2003; Wallace et al., 2006) suggest that circulating estrogens positively influence the exploration and discrimination of novel objects in females. Other studies using delays of 24 hours or more also found facilitating effects of administering estradiol, progesterone and their major metabolites, and even dihydrotestosterone and other non-aromatizable testosterone metabolites immediately after the

sample trial on subsequent novel object recognition in female subjects (Frye and Lacey, 2001; Gresack and Frick, 2006; Walf et al., 2006). Our findings provide evidence that gonadal hormones are also important in the NOR task in males and that androgens may play especially critical roles in its modulation in this sex. Further, our findings of abnormally low values of the DI with GDX that are attenuated by replacement with TP but not E are an intriguing fit with clinical evidence of diminished recognition memory in cases of hypogonadism (Salminen et al., 2004; Salminen et al., 2005) and NOR deficits of schizophrenia in male patients (Heckers et al., 2000), which are a part of the cluster of negative, prefrontal cortically mediated symptoms in this disease for which severity has also been directly linked to decreased circulating testosterone levels (Akhondzadeh et al., 2006; Goyal et al., 2004; Huber et al., 2005; Ko et al., 2007; Shirayama et al., 2002; Taherianfard and Shariaty, 2004).

Summary and Conclusions.

The findings from this study suggest that in adult male rats, gonadal hormones influence mnemonic functions associated not only with the medial and orbital, but also with the perirhinal division of the prefrontal cortex. Like effects on the spatial working memory processes of the medial prefrontal cortex demonstrated previously (Daniel et al., 2003; Kritzer et al., 2007; Kritzer et al., 2001), the adverse effects of GDX on the NOR task observed here were attenuated by supplementing GDX animals with TP but not E, and were significantly correlated with a bioassay of

circulating androgens-- BSM weight. Although not examined in this study, there are several reasons to suspect that correlations previously found between the effects of GDX on medial prefrontal working memory function and on the density of its functionally critical dopamine (DA) innervation (Kritzer et al., 2007) may also extend to the perirhinal-dependent processes of the NOR task, since the effects of GDX on DA innervation in perirhinal prefrontal fields are qualitatively and quantitatively similar to those observed in medial prefrontal areas and are likewise androgen dependent (Kritzer et al., 2003), and because performance on NOR tasks, like that of the medial prefrontal cortical-mediated spatial working memory (Stam et al., 1989; Zahrt et al., 1997) is also impaired by selective perturbations of the prefrontal dopamine system (Morrow et al., 2000). Accordingly, as the study of gonadal hormone influence in males over the executive and mnemonic functions of the prefrontal cortex in rats continues to expand, it may be increasingly important to explore the DAergic endpoints in parallel with behavior. Therefore, in the next chapter of this dissertation, the pivotal endpoint of extracellular DA level will be addressed in this same GDX animal model.

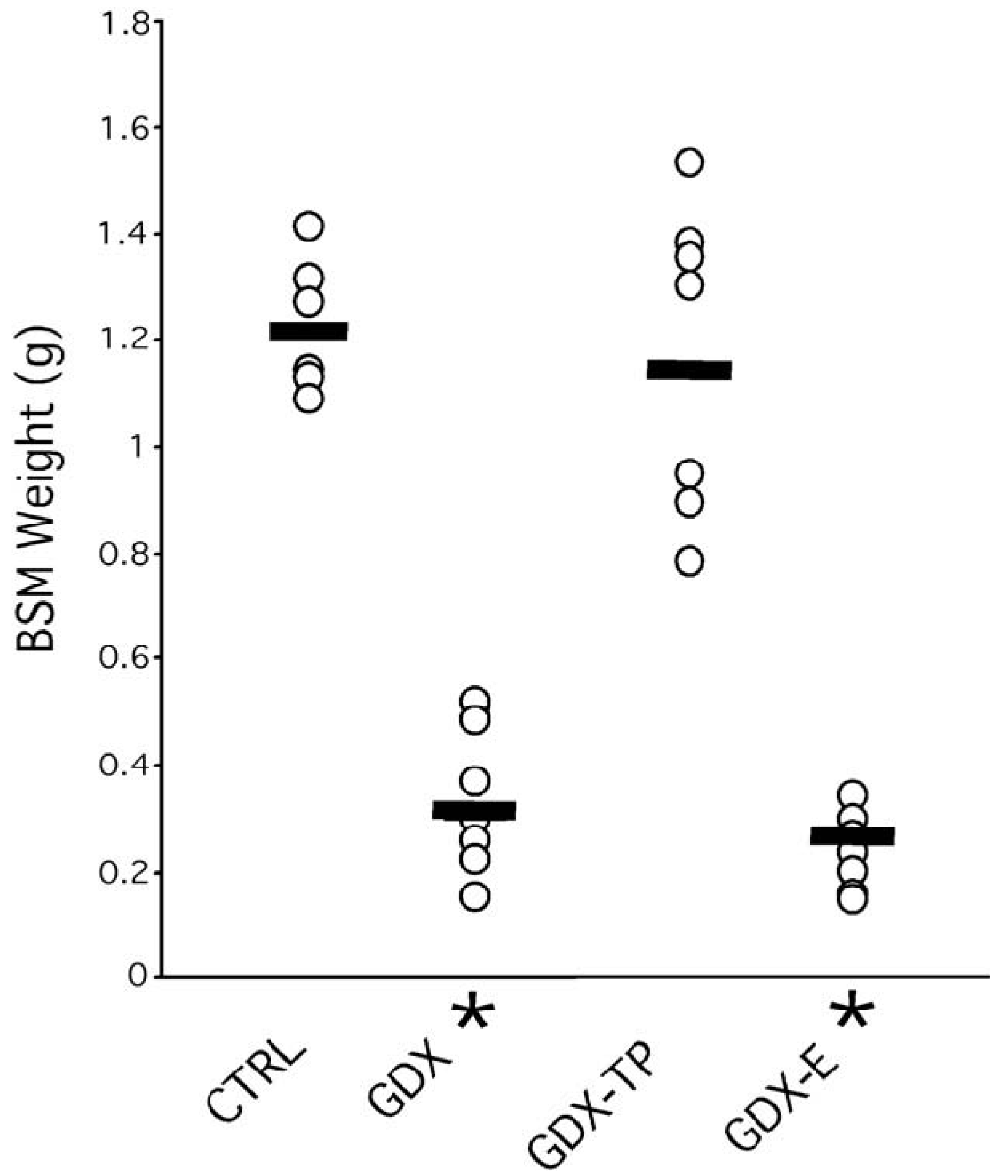

Figure I.1. Scatterplots showing bulbospongiosus muscle (BSM) weights in grams (g) of individual animals (dots). Horizontal lines show the average weight for each group. As expected, BSM weights in gonadectomized (GDX) rats and in GDX rats given estradiol (GDX-E) were significantly lower (*) than weights in sham-operated (CTRL) and GDX rats given testosterone propionate (GDX-TP).

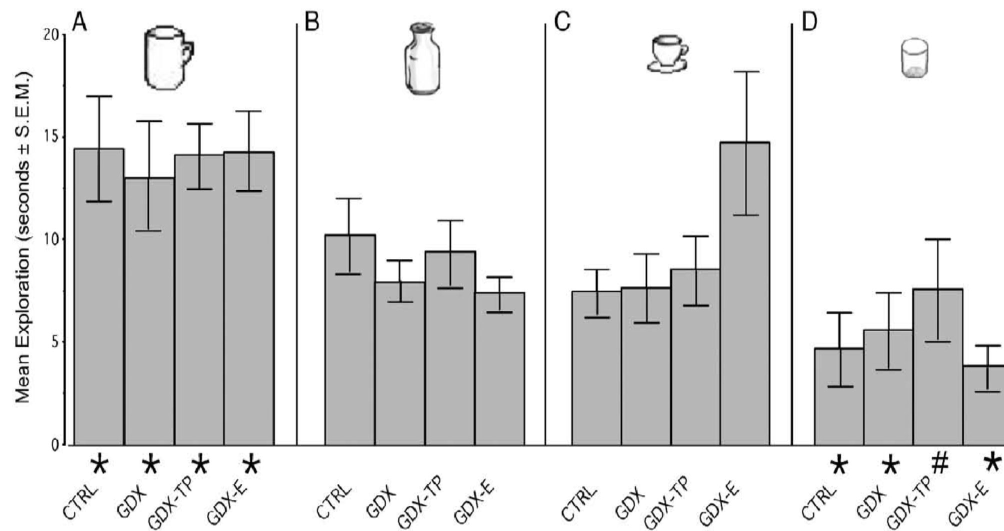

Figure I.2. Bar graph showing the mean amount of time (s) animals explored each of four sample objects present in the testing field. Control (CTRL), gonadectomized (GDX), and GDX rats given testosterone propionate (GDX-TP) or estradiol (GDX-E) all spent significantly (*) more time with large/complex objects (A), significantly to near significantly (#, $p<0.07$) less time with small/smooth objects (D), and intermediate amounts of time with large/smooth (B) and small/complex (C) objects.

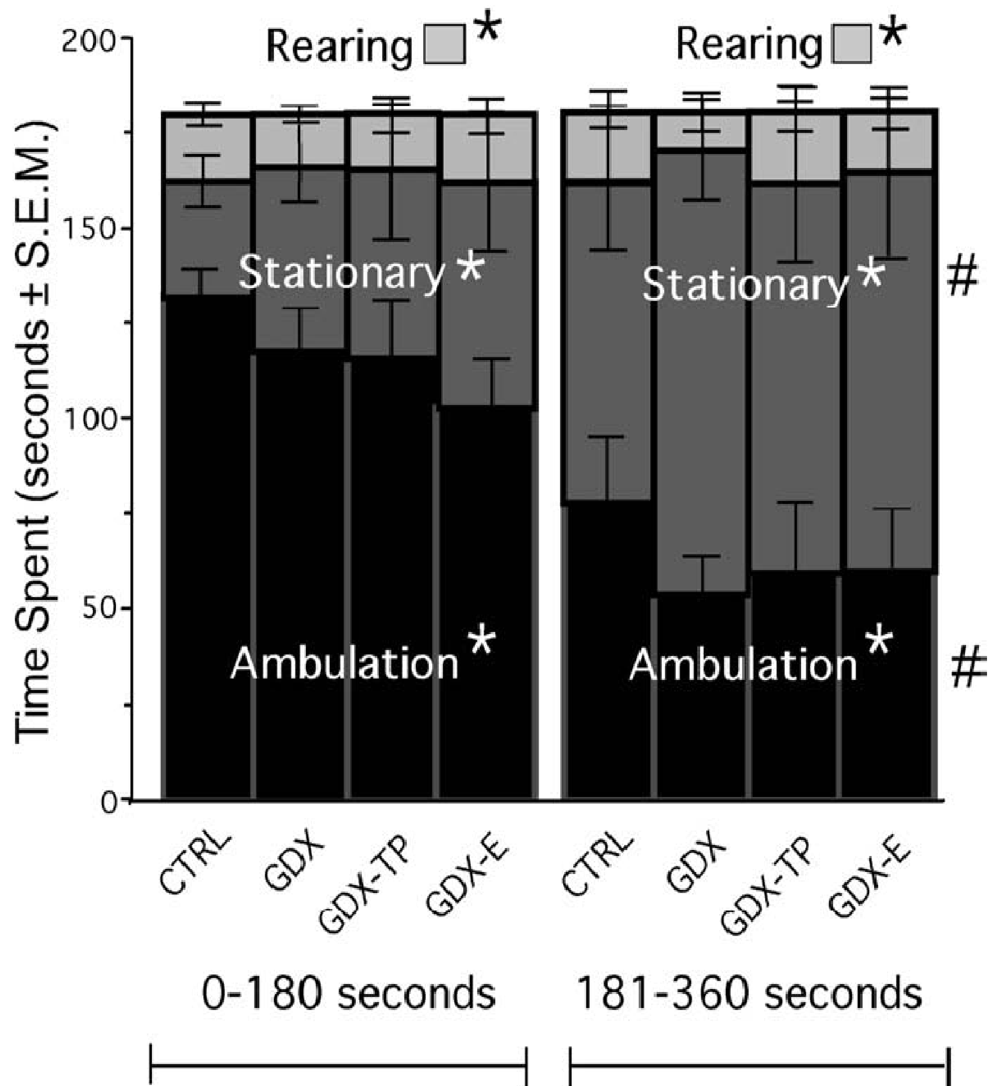

Figure I.3. Stacked bar graphs showing the mean amounts of time spent ambulating (black), stationary (dark grey) and rearing (light grey) during the first and final 180 s of a 6 min open-field session for each group. For control (CTRL), gonadectomized (GDX), and GDX rats given testosterone propionate (GDX-TP) or estradiol (GDX-E), significantly different amounts of time were devoted to each principal behavior (*). In addition, during the second half of the session all groups spent significantly (#) more time stationary and less time ambulating than initially. However, there were no significant main effects of hormone treatment or significant interactions between hormone treatment and behavior.

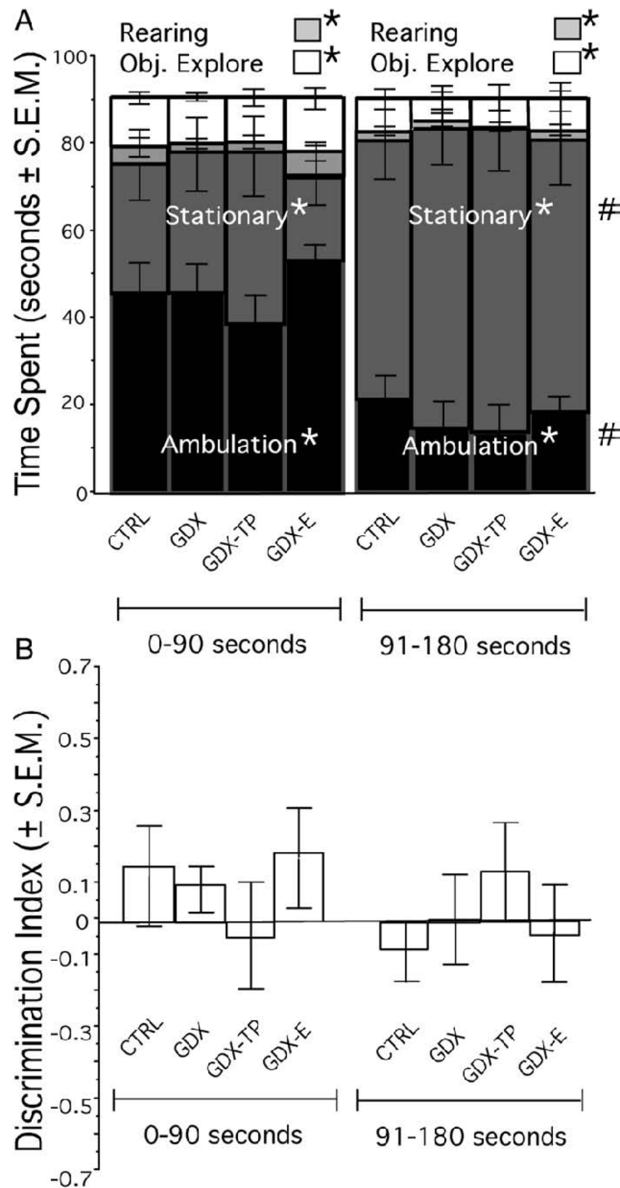

Figure I.4. A) Stacked bar graphs showing the mean amounts of time spent ambulating (black), stationary (dark grey), rearing (light grey) and exploring objects (white) during the first and final 90 s of a 3 min object recognition task habitation session for each group. For control (CTRL), gonadectomized (GDX), and GDX rats given testosterone propionate (GDX-TP) or estradiol (GDX-E) significantly different amounts of time were devoted to each principal behavior (*). During the second half of the session all groups also spent significantly (#) more time stationary and less time ambulating than initially. No significant main effects of hormone treatment or significant interactions between hormone treatment and object exploration were found. B) Bar graphs showing the Discrimination Index (DI) of object exploration during the first and second halves of the object recognition task habitation trial. For all groups DI values were consistently near zero, indicating that all groups interacted with the two identical objects present for similar amounts of time.

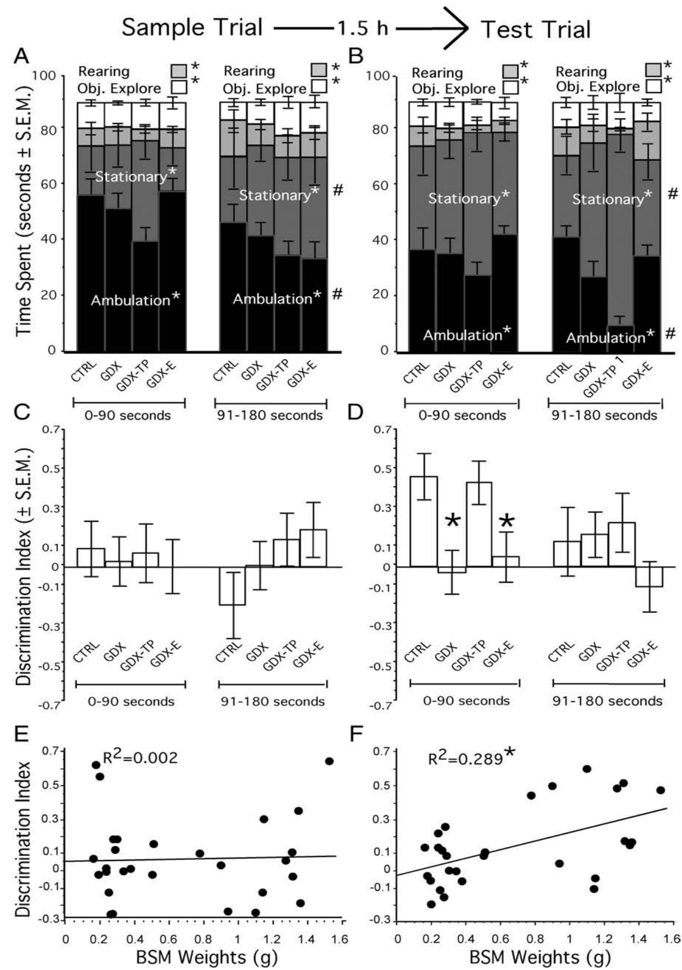

Figure I.5. Behavioral data collected during the sample (A, C, E) and test (B, D, F) trials of the spontaneous novel object recognition task with the 1.5 h delay. Stacked bar graphs showing the mean amounts of time spent ambulating (black), stationary (dark grey), rearing (light grey) and exploring objects (white) during the first and final 90 s of a 3 min sample trial (A) and test trial (B) show that the control (CTRL), gonadectomized (GDX), and GDX rats given testosterone propionate (GDX-TP) or estradiol (GDX-E) spent significantly (*) different amounts of time on each principal behavior and that during the second half of the session all groups spent significantly (#) more time stationary and less time ambulating than initially. During the test phase only (B) significant main effects of hormone treatment were observed that were driven by the ambulatory and stationary behaviors of the GDX-TP group (1). Bar graphs showing the Discrimination Index (DI) of object exploration during the first and second halves of the sample (C) and test (D) trials show that all groups explored both sample objects equally (C) and that the CTRL and GDX-TP groups spent significantly (*) more time with the novel object during the first half of the test trial (D) while the GDX and GDX-E groups did not (D). Regression plots graphing individual DI values as a function of bulbospongiosus muscle (BSM) weight in grams (g) show no correlation between the two for the sample trials (E), but a significant (*) correlation between low BSM weight and low DI values/poor object discrimination during the test trial (F).

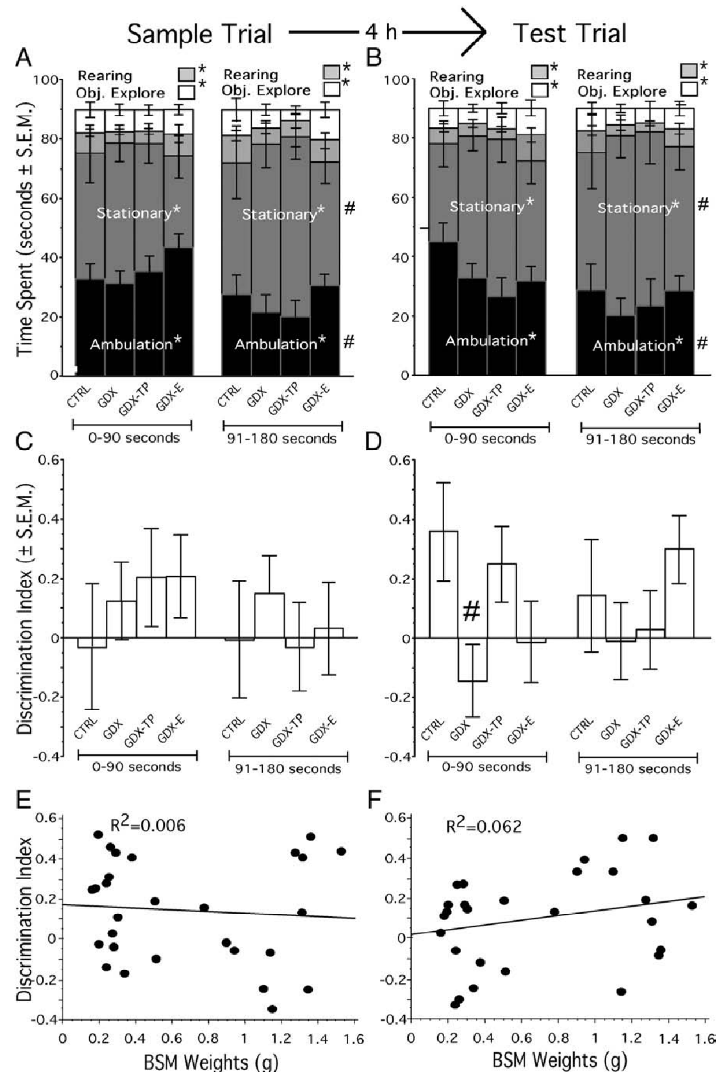

Figure I.6. Behavioral data collected during the sample (A, C, E) and test (B, D, F) trials of the spontaneous novel object recognition task with a 4 h delay. Stacked bar graphs showing the mean amounts of time spent ambulating (black), stationary (dark grey), rearing (light grey) and exploring objects (white) during the first and final 90 s of a 3 min sample trial (A) and test trial (B) show that the control (CTRL), gonadectomized (GDX), and GDX rats given testosterone propionate (GDX-TP) or estradiol (GDX-E) spent significantly (*) different amounts of time on each principal behavior and that during the second half of the session all animal groups spent significantly (#) more time stationary and less time ambulating than initially. Bar graphs showing the Discrimination Index (DI) of object exploration during the first and second halves of the sample (C) and test (D) object recognition trials show that all animal groups explored both sample objects equally (C) and that the CTRL and GDX-TP groups spent more time with the novel object during the test trial (D) while the GDX and GDX-E groups spent similar amounts of time with the two objects (D; #, GDX compared to CTRL p=0.072). Regression plots graphing individual DI values as a function of bulbospongiosus muscle (BSM) weight in grams (g) show no correlation between the two for the sample trial (E) and a weak relationship between low BSM weight and impaired object recognition in the test trial (F).

Chapter II

Gonadectomy and hormone replacement affects *in vivo* basal extracellular dopamine levels in the prefrontal cortex but not motor cortex of adult male rats

As discussed previously, the prefrontal cortices (PFC) are important mediators of highest order information processing and controllers of functions such as working memory and behavioral flexibility (Goldman-Rakic et al., 1990; Dalley et al., 2004). These complex functions are known to be exquisitely sensitive to local dopamine (DA) signaling, and information from gene polymorphisms and disease processes in humans (Davis et al., 1991; Goldberg et al., 2003) and from studies in experimental animal models (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Morrow et al., 2000) sum to suggest that both increased and decreased DA tone can adversely affect DA-dependent PFC function. In rats, for example, chemical lesions of PFC DA afferents (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Stam et al., 1989), local administration of dopamine D1 receptor agonists and antagonists (Zahrt et al., 1997; Winter et al., 2009) and NMDA receptor antagonists (Seamans et al., 1995; Verma and Moghaddam, 1996) as well as β-carboline- and stress-induced increases in DA turnover (Murphy et al., 1996; Morrow et al., 2000; Moghaddam and Jackson, 2004) all adversely affect performance in frontal lobe-dependent tasks including open field testing, delayed alternation paradigms and novel

object recognition. Given the more recent evidence showing that performance on these same DA-dependent PFC tasks is also sensitive to gonadal hormones (Einon, 1980; van Haaren et al., 1990; Janowsky et al., 2000; Lacreuse, 2006; Luine, 2008), the question arises as to whether these behavioral influences of gonadal steroids are related to their modulation of PFC DA signaling. To begin to address this, methods of *in vivo* microdialysis were paired with gonadectomy and hormone replacement in adult male rats to determine whether these hormone manipulations affect the functionally pivotal metric of basal PFC DA tone.

Although assessments of sex differences (Dawson et al., 1975; Einon, 1980; van Hest et al., 1988; Overman, 2004; Shansky et al., 2004; Stanton et al., 2009) and of ovarian hormone influence in females (Rapp et al., 2003; Wide et al., 2004; Sinopoli et al., 2006; van Wingen et al., 2007; Frye and Walf, 2008; Hatta and Nagaya, 2009) are more numerous, a growing number of studies demonstrate significant hormone sensitivity of PFC function in males. In humans, for example, positive correlations have been identified between circulating testosterone level and PFC function in healthy subjects while negative correlations have been observed between testosterone titers and the severity of cognitive deficits in aging (Janowsky, 2006), androgen deprivation therapies (Nelson et al., 2007) and in disorders such as schizophrenia (Shirayama et al., 2002; Goyal et al., 2004; Taherianfard and Shariaty, 2004; Akhondzadeh et al., 2006; Ko et al., 2007). In adult male rats, gonadectomy (GDX) and/or

hormone replacement has also been shown to significantly impair acquisition (Ceccarelli et al., 2001; Kritzer et al., 2001; Daniel et al., 2003; Kritzer et al., 2007) and/or negatively impact performance (Adler et al., 1999; Sandstrom et al., 2006; Aubele et al., 2008; Gibbs and Johnson, 2008) in open field testing, maze and operant-versions of the spatial delayed alternation task, extradimensional set-shifting/behavioral flexibility and novel object recognition—which are all tasks that are well known to be sensitive to PFC lesions (Bubser and Schmidt, 1990; Barker et al., 2007; Naneix et al., 2009; Tait et al., 2009) and to selective mesoprefrontal DA perturbations (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Stam et al., 1989; Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Morrow et al., 2000).

The possibility that the effects of GDX on these tasks are linked to hormone actions on the mesoprefrontal DA system is suggested first by findings that GDX selectively affects both PFC function and PFC DA axon density in an androgen-sensitive, estrogen-insensitive manner (Kritzer et al., 1999; Kritzer, 2000; Kritzer et al., 2001; Aubele et al., 2008) and further in studies showing that for several tasks these functional and axon density effects are significantly correlated to one another (Kritzer et al., 2007). However, because the endpoint of innervation density may not reflect a functionally relevant index of PFC DA signaling, the studies presented here used *in vivo* microdialysis to ask whether GDX and/or hormone replacement might also affect basal PFC DA tone. Thus, using

the same adult male rat models used in previous behavioral studies (Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008) and/or anatomical analyses of cortical DA axon density (Kritzer et al., 1999; Kritzer, 2000) basal extracellular DA level was measured in the medial PFC of rats that had been sham operated, gonadectomized or gonadectomized and supplemented with testosterone propionate or estradiol for 4 or 28 days-- time points where GDX is known to significantly decrease and increase PFC DA axon density, respectively. For comparison, extracellular DA level was also measured in the nearby and DA-enriched motor cortical fields of separate cohorts of 4- and 28-day GDX and control animals.

Methods

Animal Subjects: A total of 64 adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Animals were housed in pairs of like treatment under a 12/12 hour light/dark cycle with food and water available *ad libitum.* Twenty animals were sham-operated (CTRL); 9 were used in experiments 4 days after surgery, and the remainder were used after 28 days. The remaining 44 animals were gonadectomized (GDX); 9 subjects made up the 4-day GDX cohort and 12 were used in the 28-day GDX group. Thirteen GDX rats were supplemented with testosterone propionate (GDX-TP); 5 were used for the 4 day cohort, and 8 were used in the 28 day GDX-TP group. Ten GDX animals were supplemented with

17-β-estradiol, 5 animals were used in the 4-day GDX-E cohort and 5 were used in the 28-day GDX-E group. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize their use and discomfort.

Surgeries: All surgeries were carried out under aseptic conditions and used intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. Rats were monitored during recovery from surgery and given 0.03 mg/kg buprenorphine to manage any post-operative discomfort.

Sham surgery or gonadectomy was performed 4 or 28 days before microdialysis. For both procedures, the sac of the scrotum and the underlying layers of tunica were incised. For GDX, the vas deferens was also bilaterally ligated and the testes removed. Incisions were closed using sterile wound clips that were removed two weeks later for the 28-day survival animals.

Craniotomy was performed for placement of microdialysis probe guide cannulae on the day prior to the microdialysis experiment. For this procedure, anesthetized rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA), an incision was made to expose the skull and burr holes were drilled for inserting and anchoring guide cannulae (CMA Microdialysis, North Chelmsford, MA, USA) to the skull. Using coordinates adapted from the atlas of Paxinos and Watson (1998), cannulae were

aimed toward either the left pregenual medial prefrontal cortex or left primary motor cortex located 3.2 mm anterior to Bregma, and were secured with a combination of shallow screws and dental cement.

Hormone Supplements: For the hormone-supplemented groups, pellets that released either 3–4 ng of TP (GDX-TP rats) or 25 pg of E per milliliter of blood per day (GDX-E rats, Innovative Research of America, Sarasota, FL) were inserted within the tunica at the time of GDX. These pellets and doses have been used in previous investigations in this laboratory and others and have been shown to yield circulating level of gonadal hormones that fall within the physiological range (Collins et al., 1992; Adler et al., 1999; Kritzer, 2000).

In Vivo Microdialysis: On the morning after craniotomy, awake, freely-moving animals were placed in Raturn clear rodent bowls (BioAnalytical Systems, West Lafayette, IN, USA) and allowed to acclimate for 30 minutes before microdialysis probes (100,000 Dalton cut-off, 2mm PES exposed membrane tip, CMA Microdialysis, North Chelmsford, MA, USA) were slowly lowered through guide cannulae into place. For the next 2 hours, the probes were perfused with artificial CSF (145 mM 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 H2O, pH 6.8) at a rate of 2 μL/min, during which time subjects fell asleep. Afterward, dialysates were collected every 20 minutes from PFC and/or motor cortex. This phase of the experiment was between 0900-1800 hours which corresponded to the rats' subjective night. At least

3 samples were collected from each animal at times when they were observed to be sleeping; for the very few cases where a given 20 minute sample was collected while an animal was transiently awake, that sample as well as the one collected over the next 20 minutes were excluded from the analysis due to the increases in cortical DA levels that are known to occur with arousal and movement. Dialysate samples (5µL) were directly injected into an HPLC system (PM 92-E pump, BAS, West Lafayette, IN) via an on-line autoinjector (Pollen-8, BAS, West Lafayette, IN). Sample analyses utilized a microbore column (UniJet, 1.0 mm inner diameter, 150mm length, 5 µm ODS particles; BAS, West Lafayette, IN, USA) and a BioAnalytical Systems LC-Epsilon detector (BAS, West Lafayette, IN, USA); the E_{app} was + 0.65 V versus the Ag/AgCl reference electrode and 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; .027 mM EDTA; 7.2% acetonitrile (v/v), 1% tetrahydrofuran (v/v), pH 3.4. Probe efficiency was measured at 12-18% for all studies, and an overall detection limit of 10 fmol was achieved. All chemicals used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Euthanasia and Histology: At the conclusion of the microdialysis study, animals were euthanized via rapid decapitation and their brains were removed and immersed in a 30% sucrose, 10% buffered formaldehyde solution for fixation and cryoprotection. The androgen-sensitive medial ventral and lateral bulbospongiosus muscles (BSM) were also dissected

out and weighed. After the brains sank in the sucrose solution, the frontal cortex region of each subject was blocked, rapidly frozen and serially sectioned in the coronal plane on a freezing microtome at a thickness 40 μm. For each animal subject, a 1 in 4 series of sections taken from mid olfactory bulb through the genu of the corpus callosum was collected, slide mounted, Nissl stained, and examined to verify the cytoarchitectonic location of the microdialysis probe. In order for data from a given animal to be included in the analyses, the following criteria had to be met: for the PFC, probe tips had to be placed in the left pregenual medial PFC, extend dorsoventrally through prelimbic and infralimbic areas but not beyond to dorsopeduncular cortex, and be centered over middle and deep cortical layers (see Figure II.1); with this placement, the 2mm probe tip length covered about 1mm in each of these two medial prefrontal fields. For studies in motor cortex, probes had to be placed on the lateral cortical surface within the left premotor (M2) and/or primary motor (M1) field and not extend beyond into white matter, claustrum, or insular cortex (AID, AIV) (see Figure II.2).

<u>Data analysis</u>: Dopamine peak identity was confirmed and quantified (ng/10 mL) in relation to standard peaks of known concentrations (1, 2, 5, 10 ng/10mL) run through the HPLC system on the same day as the animal data were collected; uncorrected peak values were measured automatically by ChromGraph software (BAS, West Lafayette, IN, USA) and peak heights and were converted to concentrations (fmol/μL) using

the standards. Because DA metabolites such as homovanillic acid (HVA) and 3, 4 dihydroxyphenlyacetic acid (DOPAC) were detected in only a subset of the animals, these were not assessed. Basal DA levels were averaged per subject from at least 3 separate 20-minute dialysate samples. Average values from individuals were then compiled and assessed by group using descriptive statistics followed by one-way analyses of variance (ANOVA) to probe for significant main effects of hormone treatment on basal DA measures per cortical region. Allowed post-hoc testing used the Fisher's Protected Least Significant Difference (PLSD), and in all cases $p< 0.05$ was accepted as significant. BSM weights were also assessed using the same descriptive and comparative statistical methods, and regression analyses in which the BSM weights were used as independent variables and the individual animals' extracellular PFC DA (above) were used as dependent variables were also carried out.

Results

Effectiveness of Hormone Treatments

The weights of the androgen-sensitive bulbospongiosus muscles (BSM) showed expected group differences among both the 4- and 28-day treatment groups. Specifically, in both cases the mean muscle weights of the CTRL and GDX-TP rats were higher than those of the GDX and GDX-E cohorts. However, as expected as well, the between group differences

in muscle weights were more pronounced for the 28 compared to the 4 day treatment groups (see Figure II.3). Nonetheless, separate analyses of variance that compared individual animal BSM weights revealed significant main effects of hormone treatment for both the longer and shorter treatment groups [4 day: $F(3, 15) = 10.471$, $p = 0.0006$; 28 day: $F(3, 23) = 339.432$, $p < 0.0001$]. Allowed post-hoc tests further confirmed that for both sets of animals, the BSM weights of the control and GDX-TP cohorts were similar to and not significantly different from one another, the weights of the GDX and GDX-E rats were similar to and not significantly different from each other, and that the BSM weights of the 4 and 28 day GDX and GDX-E cohorts were both significantly different from those of the corresponding control and GDX-TP rats (Figure II.3).

Extracellular Prefrontal Cortical Dopamine Level

4 -Day Treatment Groups: Extracellular levels of DA measured in the left pregenual medial PFC of the 4-day sham-operated (CTRL) rats were similar to values previously reported in *in vivo* microdialysis studies of DA in the medial PFC of adult male rats (Moghaddam and Jackson, 2004; Stefani and Moghaddam, 2005; Del Arco et al., 2007; van der Meulen et al., 2007; Balla et al., 2009). Thus, values in the CTRL group ranged from 0.10-0.23 fmol/µL and had an average of 0.15 ± 0.02 fmol/µL. For the GDX cohort however, basal extracellular PFC DA level was roughly 40% lower (range of 0.08 - 0.11 fmol/µL; mean of 0.09± 0.004 fmol/µL) while PFC DA level in both hormone-supplemented groups

(GDX-TP and GDX-E rats) was similar to controls; (GDX-TP: range of 0.11 - 0.21 fmol/μL; mean 0.16 ± 0.02 fmol/μL, GDX-E: range of 0.13 – 0.18 fmol/μL; mean 0.15 ± 0.01 fmol/μL, see Figure II.4A). Statistical evaluation (analyses of variance) of these data further identified significant main effects of hormone treatment on basal PFC DA level [$F_{(3, 15)} = 4.168$, $p = .025$], and post-hoc comparisons confirmed that basal PFC DA values in the GDX-TP, GDX-E and CTRL groups were all similar and not significantly different from one another, and that the extracellular PFC DA level in the GDX rats was significantly lower than those of the other treatment groups (vs. CTRL, $p < 0.0094$; vs. GDX-TP, $p<0.0086$; vs. GDX-E, $p<0.0254$). Because this effect was attenuated by replacement with both testosterone and estrogen and thus included animals with both higher and lower BSM weights, regression analyses assessing animals' individual and average measures of extracellular PFC DA level as a function of BSM weight found no significant correlations (individual: $R^2 = 0.088$, $p < 0.108$ average: $R^2 = 0.115$, $p < 0.0856$) between PFC DA and androgen-sensitive muscle weight (Figure II.4B).

28 -Day Treatment Groups: Basal, extracellular DA levels measured in the 28-day CTRL group were similar to those obtained in the 4-day CTRL rats and to DA level reported in previous *in vivo* microdialysis studies of PFC in adult male rats (Moghaddam and Jackson, 2004; Stefani and Moghaddam, 2005; Del Arco et al., 2007; van der Meulen et al., 2007; Balla et al., 2009); these values were between 0.08 and 0.18 fmol/μL and

were on average 0.13 ± 0.04 fmol/μL. Basal DA level measured in the 28-day GDX cohort, however, was nearly twice as high as control, ranging from 0.18 to 0.30 fmol/μL and averaging 0.23 ± 0.02 fmol/μL. Similarly elevated DA level was also observed in GDX-E rats (range of from 0.16 - 0.27 fmol/μL; mean 0.22 ± 0.02 fmol/μL, see Figure II.4C) while the values in the GDX-TP rats were similar to control; (range of 0.09 - 0.15 fmol/μL; mean 0.12 ± 0.01 fmol/μL, see Figure II.4C). Analyses of variance performed on these data identified significant main effects of hormone treatment on mean PFC DA level [F (3, 24) = 9.197, p = .0003], and allowed post-hoc comparisons confirmed that basal PFC DA level in the GDX and GDX-E but not the GDX-TP group was significantly higher than control (CTRL vs. GDX, p<0.0017; CTRL vs. GDX-E; p<0.0116). In keeping with the selective androgen sensitivity of the GDX effect, regression analyses assessing animals' individual and mean measures of extracellular PFC DA level as a function of BSM weight further identified significant correlations between both measures of PFC DA level and androgen-sensitive muscle weight (individual: R^2 = 0.249, p < 0.001 average: R^2 = 0.528, p < 0.001, see Figure II.4D).

Extracellular Motor Cortex Dopamine Level

For comparison, the effects of GDX were also assessed in a second DA-enriched area of the rat cerebrum-- the motor cortical fields. However, in both 4 and 28-day sham-operated control and GDX rats alike, basal DA levels in this region were all between 0.09 - 0.23 fmol/uL and

had mean values that ranged from 0.14 - 0.17 fmol/uL (Figure II.5 A, B). Analyses of variance found no significant main effects of hormone treatment on these motor cortex measures, and regression analyses assessing animals' individual and mean measures of extracellular sensorimotor DA level as a function of BSM weight revealed no significant correlations between motor cortical DA level and androgen-sensitive muscle weight (4 day: $R^2 = 0.029$, $p < 0.314$; 28 day: $R^2 = 0.038$, $p < 0.302$, see Figure II.5 C, D). Accordingly, no assessments of motor cortex were made in GDX-hormone-supplemented animals.

Discussion

As evidence for the impact of gonadal steroid hormones on the development, adult performance and the influence of disease of the highest order cognitive, affective and mnemonic functions of the PFC grows, efforts to understand how this modulation might occur are expanding. The studies presented here follow up on findings suggesting that in males the function of the prefrontal cortices may be modulated via gonadal steroid actions on their essential mesoprefrontal DA innervation (Janowsky et al., 2000; Kritzer et al., 2001; Gibbs, 2005; Muller et al., 2005; Kritzer et al., 2007; Aubele et al., 2008). For example, these studies have foundations in previous anatomical studies from this laboratory showing striking effects of both long- and short- term GDX on medial prefrontal DA innervation density (Adler et al., 1999; Kritzer, 2000), on behavioral analyses showing GDX-induced deficits in DA- and prefrontal

cortical-dependent tasks (Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008) and on the significant correlations that have also been identified between these anatomical and behavioral effects (Kritzer et al., 2007). Here, however, it was asked whether the effects that GDX and hormone replacement have on PFC DA innervation density might be a marker for hormone effects on the potentially more functionally relevant *in vivo* measure of PFC DA level. Thus, using *in vivo* microdialysis, the effects of long- and short-term GDX and hormone replacement on basal extracellular DA level were assessed in the medial PFC and, for comparison, in the nearby and also DA-enriched motor cortical fields. In keeping with the transient, estrogen-sensitive decreases in PFC DA axon density that have been observed following short term GDX (Kritzer, 2000), extracellular PFC DA level was found to be significantly lower than control in the 4-day GDX cohort and similar to control in 4-day GDX animals supplemented with both TP and with E.

Further, just as long-term GDX has been shown to increase PFC DA axon density in an androgen-sensitive, estrogen-insensitive manner (Kritzer, 2000, 2003), extracellular PFC DA level was also found to be significantly higher than control in the 28-Day GDX and GDX-E cohorts and similar to control in 28-day GDX animals supplemented with TP. At neither time point, however, did the extracellular DA level in the motor cortical fields of GDX animals differ significantly from control. Although the endocrine mediators governing the temporally and spatially complex

responses that have now been observed in this model for several DAergic PFC endpoints remain unknown, it is tempting to speculate that hormone actions at intracellular estrogen and androgen receptors located either within the prefrontal cortex itself (Kritzer, 2002, 2006) or within mesoprefrontally projecting midbrain DA neurons (Kritzer and Creutz, 2008) could be involved. For example, estrogen in the brain has the capacity to use rapid non-genomic signaling and can downregulate its cognate receptors over time (Lauber et al., 1990; Simerly and Young, 1991; Toran-Allerand, 1991); these abilities could explain the initial estrogen-sensitivity and later estrogen-insensitivity that is observed for both PFC DA level and DA axon density following GDX. Further, the differential consequences that supplementing GDX animals with estrogen vs. testosterone has for PFC DA levels and axon density are also in keeping with the many examples of opposing influences that have been found for ovarian vs. testicular hormone/intracellular receptor interactions in brain and peripheral tissues (Toran-Allerand, 1991; Stewart and Rajabi, 1994; Handa et al., 1997; Cutolo and Wilder, 2000; Zhang et al., 2000; Krause et al., 2006). While the question of how they arise remains pure speculation, it is clear that the initial, short-term effects of GDX on PFC DA systems including those identified in this study are transient while those that are observed later at 28 days post GDX appear to be more stable. Because effects at this longer time point also represent the condition where behavioral effects of GDX on DA-dependent prefrontal functions

have been found (Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008), discussion in the sections that follow focus mainly on results pertaining to the 28-day animal groups. The striking and seemingly PFC-selective effects of GDX on DA level that are seen at this stage are first compared to results from previous studies of sex differences and/or hormone effects on cortical DA concentration are then discussed in terms of possible mechanism and are finally considered with respect to the sex differences and hormone modifiability that are known to characterize information processing in the PFC of animal models and in humans in health and disease.

Hormone Effects on Prefrontal Cortical DA Level: Comparison to Previous Studies.

Although numerous studies have found evidence for sex differences, estrus cycle variance and/or gonadal hormone regulation of DA in the rat CNS, the majority of these investigations have focused on hypothalamic (Simpkins et al., 1983; Toney and Katzenellenbogen, 1986), mesostriatal (Glick et al., 1983; Camp et al., 1986; Camp and Robinson, 1988; Di Paolo et al., 1988; van Haaren and Meyer, 1991; Becker and Rudick, 1999; Becker, 2000; Walker et al., 2006) or mesolimbic (Vermes et al., 1979; Siddiqui and Gilmore, 1988; Kuhn et al., 2001; Walker et al., 2001; Parylak et al., 2008) DA systems and on endpoints such as DA receptor binding, uptake, and/or stimulated release. On the other hand,

among the relatively few assessments that have investigated sex differences and/or hormonal regulation among mesocortical DA systems are several that focus on endpoints similar to those examined here, namely whole tissue or extracellular cortical DA level. One such study examining the effects of postnatal handling on DA level found that among the unhandled controls, DA concentrations in ventral medial PFC and insular cortex homogenates were nearly 2-fold higher in female compared to male rats (Duchesne et al., 2009) while a second study using *in vivo* microdialysis showed that extracellular DA levels in the mPFC were significantly higher in female rats in estrus compared to proestrus, were significantly lower in OVX compared to intact animals, and were restored to near control level in OVX animals supplemented with E but not P (Dazzi et al., 2007). There have also been two prior studies that examined gonadal hormone impact on cortical DA level specifically in male rats. The first showed that DA and DOPAC levels measured in parietal cortex homogenates were significantly higher in long-term GDX than control rats, although a 2-day TP replacement regimen did not attenuate these effects (Battaner et al., 1987). The second study compared levels of DA, DOPAC and the other major DA metabolite, homovanillic acid (HVA) in mPFC homogenates of control, 21-day GDX and 21-day GDX adult male rats supplemented with dihydrotestosterone propionate or with E that did or did not also experience a novel environment immediately prior to euthanasia (Handa et al., 1997). Among the home-caged controls, this study found

no differences in DA, DA metabolites or their ratios between CTRL, GDX or GDX- hormone replaced subjects. The discrepancies between these negative findings and those presented here could be due to the relatively subtle differences in duration of GDX, the dose and duration of estradiol treatment and/or the use of aromatizable vs. nonaromatizable androgens. However, it may be more likely that the critical difference is that the prior mPFC studies assessed whole tissue levels of DA measured from cortical homogenates which sum DA's intracellular and extracellular pools, whereas the present study used *in vivo* microdialysis, which exclusively reflects extracellular DA level. Thus, rather than a disparity, it is possible that the data collected across these two studies both support the argument that the effects of GDX on the mesoprefrontal DA system specifically involve an exaggerated availability of DA in the prefrontal cortical extracellular space. As discussed further below, this scenario may gain some support from additional effects that GDX and hormone replacement have recently been shown to have on one player in the regulation of extracellular DA level within the mPFC.

Hormone Effects on Prefrontal Cortical DA Level: Possible Mechanisms.

Behavioral and other studies have repeatedly shown that the cognitive, mnemonic, affective and executive functions of the PFC rely on DA level being maintained within certain limits (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Morrow et al., 2000). In

addition to showing that both too much and too little DA in the extracellular space can adversely affect PFC function, previous information from *in vivo* microdialysis (Finlay and Zigmond, 1997; Watanabe et al., 1997) and electrophysiological studies (Lavin et al., 2005) have shown that DA's essential contributions to prefrontal cortical function also require an unusually extended extracellular lifetime of this transmitter once it is released (Garris et al., 1993; Cass and Gerhardt, 1994; Garris and Wightman, 1994). In contrast to the more rapid signaling that is characteristic of the subcortical DA systems (Moghaddam, 1993; Goto and Grace, 2007), DA actions in the PFC are more paracrine in nature, i.e., they rely in part on volume transmission (Garris and Wightman, 1994; Mundorf et al., 2001) owing in part to the minimal use of the dopamine (DAT) and the norepinephrine transporter (NET) in clearing clear DA from the PFC synaptic space (Sesack et al., 1998a; Sesack et al., 1998b; Miner et al., 2003; Shen et al., 2004) and instead a greater reliance on DA's extracellular inactivation largely via the degradative enzyme catechol-O-methyltransferase (COMT) (Karoum et al., 1994; Yavich et al., 2007). However, gonadal hormone regulation/GDX dysregulation of this enzymatic catabolic pathway is unlikely to contribute to the elevated extracellular DA level observed here as quantitative analyses of the O-methylation activity of both the soluble and membrane bound forms of COMT in PFC homogenates found no significant differences in either enzyme isoform among 4 and 28-day sham-operated, GDX and GDX

hormone-replaced adult male rats (Meyers et al., 2010). An influence on the DAT is also unlikely as, *in vitro* binding assays performed on PFC homogenates using the DAT-selective ligand ^{3}H WIN 35,428 also showed that neither the K_d nor the B_{max} for this binding site was affected by 4- or 28-day GDX or hormone replacement. On the other hand, results from parallel analyses using the NET-selective ligand ^{3}H nisoxetine did find significant, TP- but not E-sensitive effects of GDX on lowering the affinity of ligand binding to the NET (Meyers and Kritzer, 2009). However, the magnitude of this potentially DA elevating effect might well be expected to be modest given the limited roles that re-uptake and uptake mechanisms play in the control of PFC DA tone overall. Rather, in view of the stimulatory effects that long-term GDX has on the density of PFC axons that are immunoreactive for the DA-synthesizing enzyme tyrosine hydroxylase (Kritzer et al. 1999, Kritzer, 2000) and given the numerous precedents set for hormone effects on DA release in subcortical centers (Castner et al., 1993; Becker and Rudick, 1999; Becker, 2000; Dluzen and McDermott, 2008), perhaps more significant contributions to the anomalous extracellular PFC DA levels that are seen could come from GDX-induced dysregulation of DA synthesis and release.

Summary and Conclusions

There is growing evidence from clinical studies and animal models suggesting that androgens influence DA-dependent PFC function in males (Christiansen and Knussmann, 1987; Gouchie and Kimura, 1991;

Ceccarelli et al., 2001; Kritzer et al., 2001; Daniel et al., 2003; Janowsky, 2006; Thilers et al., 2006; Kritzer et al., 2007; Aubele et al., 2008). The present results now suggest that this may be a consequence of androgen's regulation of the functionally pivotal parameter of PFC extracellular DA level. Thus, while it had been previously shown that long - term GDX and hormone replacement with androgen selectively influences PFC DA axon density (Kritzer et al., 1999; Kritzer, 2000) in a manner that is in parallel with and significantly correlated to GDX effects on DA-dependent PFC function/behavior (Kritzer et al., 2007), the present findings show that GDX also significantly and selectively increases extracellular PFC DA level or tone-- again, in an androgen-sensitive, estrogen-insensitive manner. It is thus tempting to consider that too much PFC DA may be a large part of what produces the observed behavioral deficits in GDX animals and to speculate further that this new role for gonadal steroid and especially androgen hormones in adult male rats could help to explain and perhaps ultimately remedy the greater vulnerability of the mesoprefrontal cortical DA systems and DA-dependent PFC processes in males afflicted with disorders such as schizophrenia and ADHD (Goodman and Stevenson, 1989; Seeman and Lang, 1990). Accordingly, while previously observed androgen-sensitive effects of GDX on NET ligand binding could make some contribution to the elevated extracellular DA level observed here, it will be important to continue to characterize both the causes and the effects of hormone stimulation on

the PFC DA systems *in vivo*. Impetus for this effort comes in part from the hope that such new information could hold for informing on-going strategies for using hormone treatments as adjunct therapies in the treatment of mental illness, including the specific use of testosterone in the treatment of male schizophrenics which has already begun to show therapeutic promise in combating the negative and cognitive problems associated with this disorder (Ko et al., 2008).

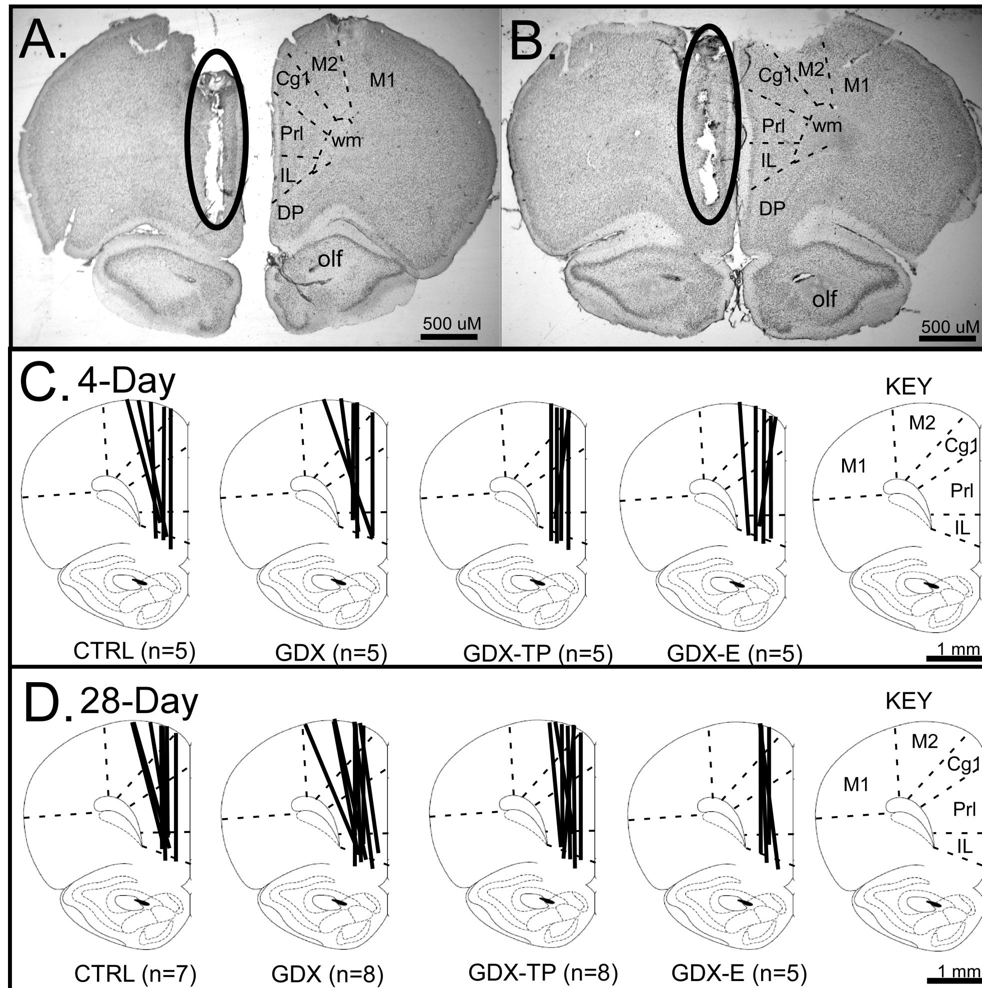

Figure II.1. Representative serial photomicrographs (A, B) showing the placement of a microdialysis probe in the medial prefrontal cortex of a control animal. Boundaries between cytoarchitectonic fields and between cortex and white matter are marked with dashed lines in the unimplanted hemisphere and damage caused by the probe itself is circled in the other hemisphere. Line drawings in panels C and D are are modified from Paxinos and Watson (1998) and illustrate the locations of microdialysis probe tracks (thick black lines) for each animal included in the 4-day (panel C) and 28 day (panel D) animal groups. For both the 4-day (C) and 28-day (D) cohorts, probes were comparably placed with respect to cortical cytoarchitecture (dashed lines) in rats that were sham-operated (CTRL), gonadectomized (GDX), gonadectomized and supplemented with testosterone propionate (GDX-TP) and gonadectomized and supplemented with estradiol (GDX-E). The number that appears in parentheses below the drawings identifies the number of animal subjects in that group. Additional abbreviations: Cg1, anterior cingulate cortex; Prl, Prelimbic cortex; IL, infralimbic cortex; DP, dorsopeduncular cortex; olf, olfactory bulb; wm, white matter.

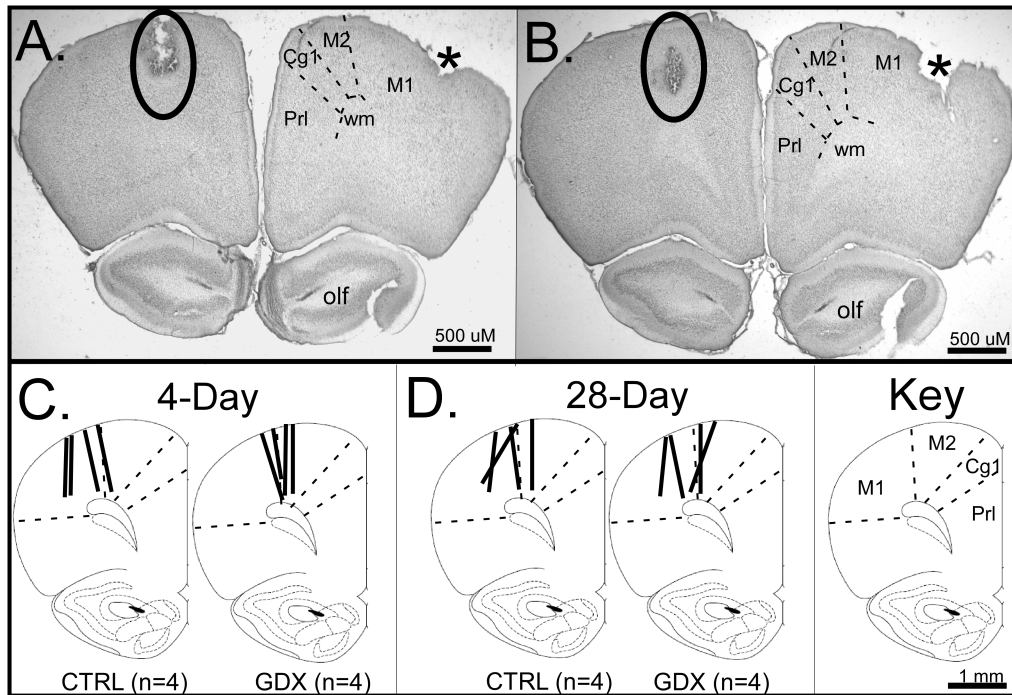

Figure II.2. Representative serial micrographs (A, B) showing the placement of a microdialysis probe in the motor cortex of a gonadectomized animal. Cytoarchitectonic fields and boundaries between cortex and white matter are marked with dashed lines in the unimplanted hemisphere, damage caused by the probe itself is circled in the other hemisphere, and the modest damage in the contralateral hemisphere caused by an anchoring screw is marked with an asterisk. Line drawings in panels C and D [modified from Paxinos and Watson (1998)] mark the locations of microdialysis probe tracks (thick black lines) for each animal in the 4-day (panel C) and 28 day (Panel D) groups. For both the 4-day and 28-day cohorts, probes were comparably placed with respect to cortical cytoarchitecture (dashed lines) in rats that were sham-operated (CTRL) and gonadectomized (GDX). The number that appears in parentheses below the drawings identifies the number of animal subjects in the group. Additional abbreviations: Cg1, anterior cingulate cortex; Prl, Prelimbic cortex; IL, infralimbic cortex; DP, dorsopeduncular cortex; olf, olfactory bulb; wm, white matter

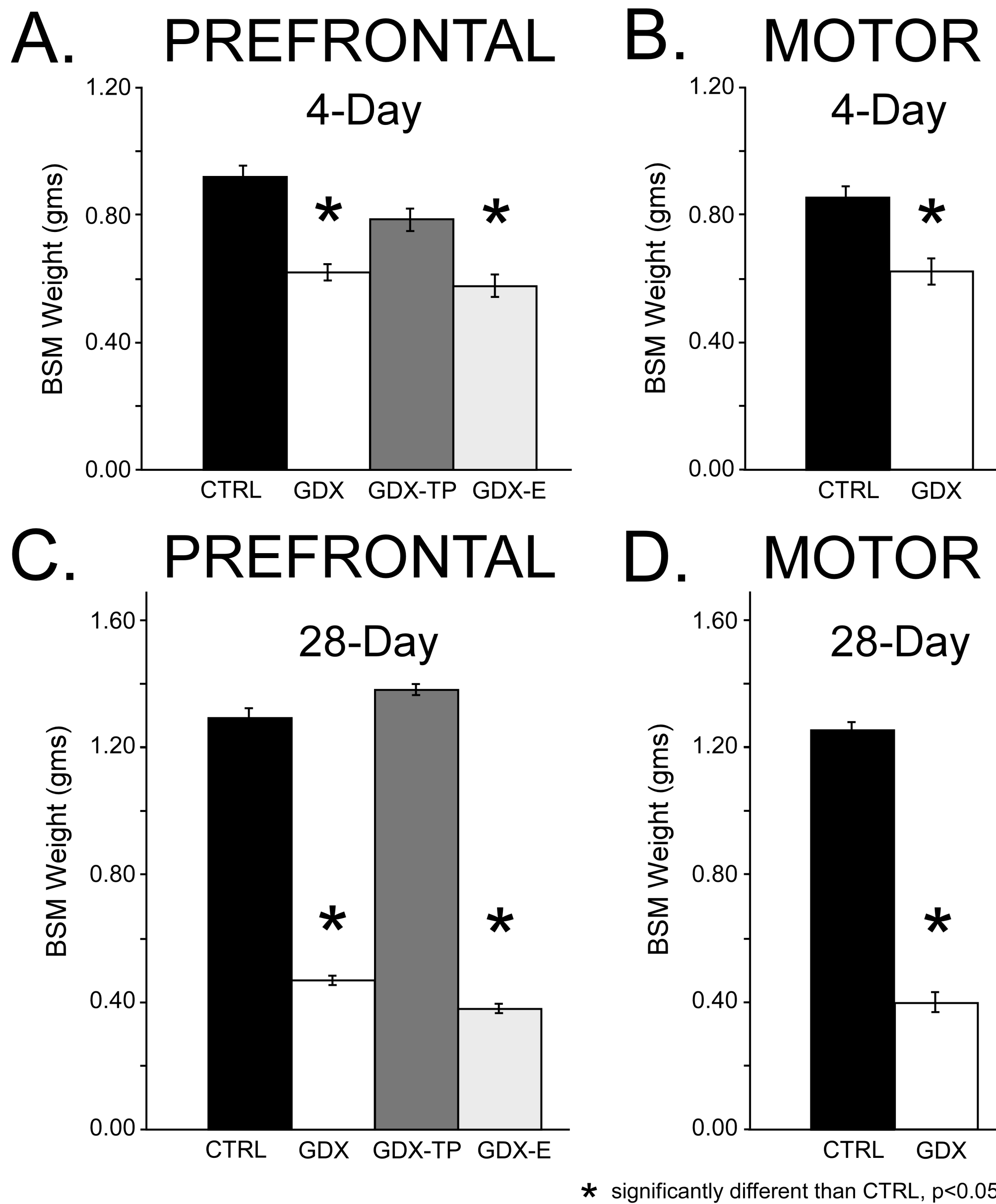

Figure II.3. Bar graphs showing mean weights of the bulbospongiosus muscles (BSM) in grams (± standard error of the mean) for animals used in microdialysis assessments of medial prefrontal cortex (A,C) and motor cortex (B, D). For the prefrontal cortex studies, data from animals that were sham-operated (CTRL, black bars), gonadectomized (GDX, white bars), gonadectomized and supplemented with testosterone propionate (GDX-TP, dark gray bars) or gonadectomized and supplemented with estradiol (GDX-E, light grey bars) for 4 days (A) or for 28 days (C) are shown separately. For the motor cortex studies, data from animals that were sham-operated (CTRL, black bars) or gonadectomized (GDX, white bars) for 4 days (B) or for 28 days (D) are also shown separately. Asterisks show that the mean weights of these androgen-sensitive muscles were significantly lower in all of the 4- and- 28 day GDX and GDX-E rats compared to the corresponding CTRL and GDX-TP groups.

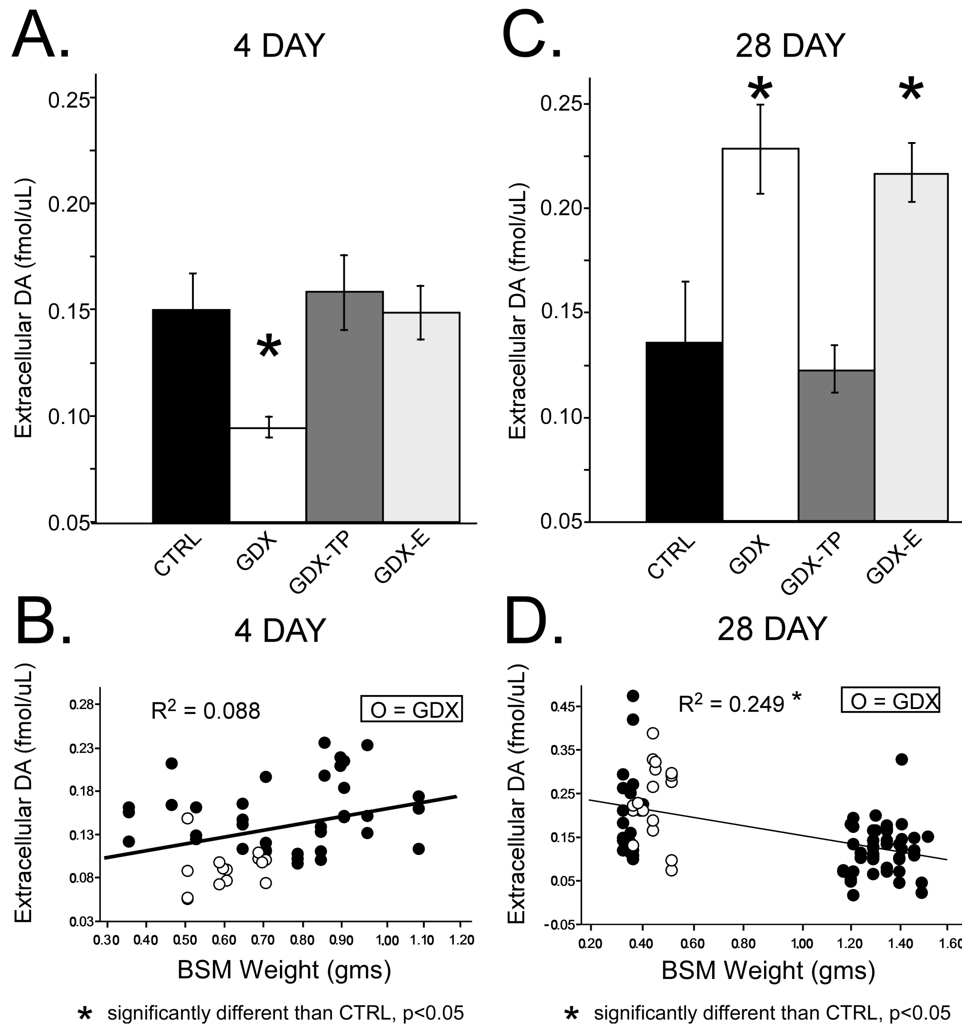

Figure II.4. Bar graphs showing mean extracellular dopamine level (DA, fmol/µL) ± standard error of the mean in 4-day (A) and 28-day prefrontal cortex (PFC, B) measured in animals that were sham-operated (CTRL, black bars), gonadectomized (GDX, white bars), gonadectomized and supplemented with testosterone propionate (GDX-TP, dark gray bars) or gonadectomized and supplemented with estradiol (GDX-E, light grey bars). Mean DA level in the PFC was significantly lower in the GDX compared to CTRL rats and was similar to control in the GDX-TP and GDX-E groups in the 4-day animals. However, in the 28-day cohort, mean dopamine level in the PFC was significantly higher in the GDX and GDX-E compared to CTRL rats and was similar to CTRL in the GDX-TP group only. Regression plots that relate 20-minute bin measurements of individual animals' level of extracellular dopamine to the weights of their androgen-sensitive bulbospongiosus muscles (BSM) in grams (gms) reveal no significant correlation between these two measures in 4-day rats (C) but that DA level and BSM weights are significantly correlated in 28-day animals (D). Open circles in the regression plots identify data points from GDX animals, and R^2 values from the regression analyses appear in the upper left.

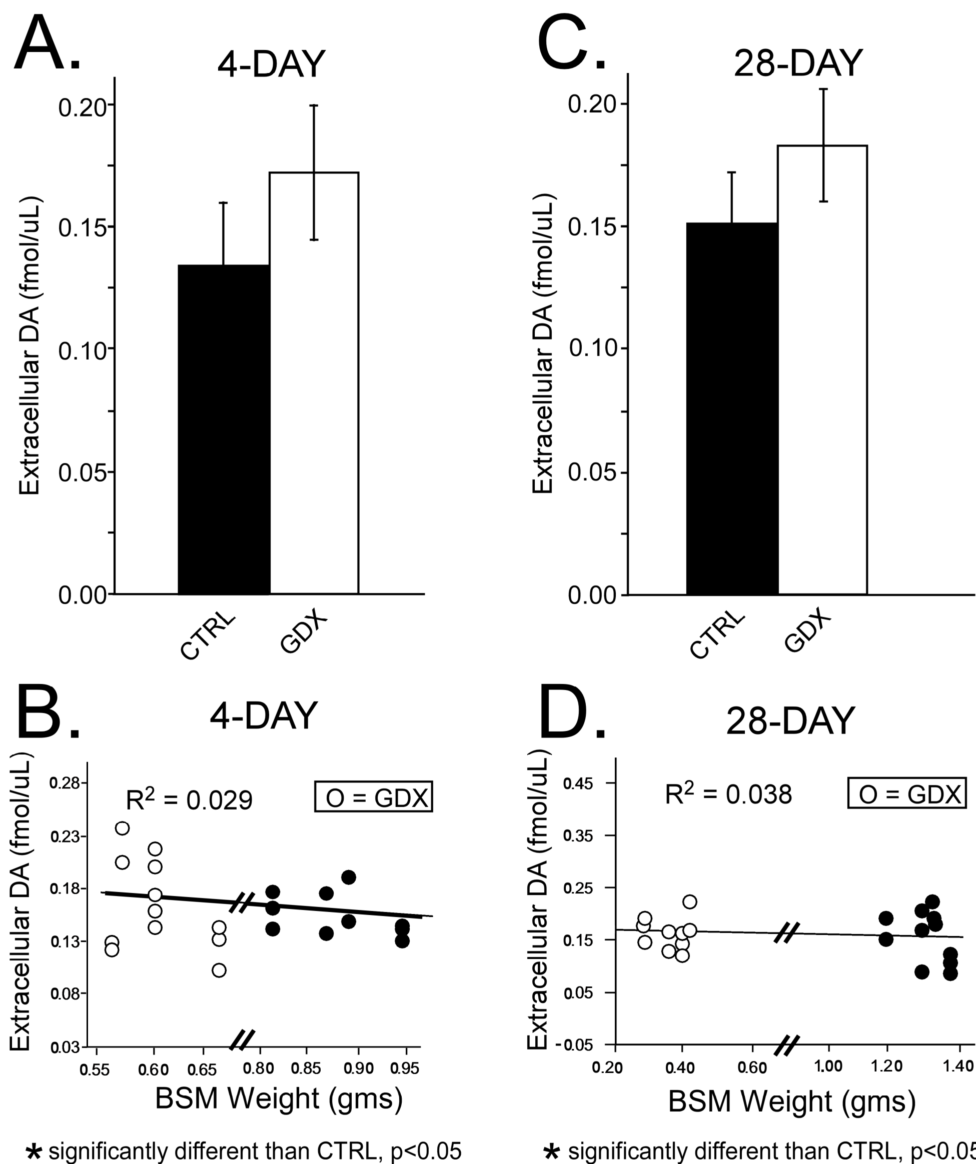

Figure II.5. Bar graphs showing mean extracellular dopamine level (DA, fmol/μL) ± standard error of the mean in 4-day (A) and 28-day motor cortex (B) measured in animals that were sham-operated (CTRL, black bars) or gonadectomized (GDX, white bars) for 28 days. There were no significant differences in extracellular DA level in motor cortex between the CTRL and GDX groups at either of these timepoints. Further, regression plots that relate 20-minute bin sample measurements of individual animals' level of extracellular dopamine to the weights of their androgen-sensitive bulbospongiosus muscles (BSM) in grams (gms) reveal no significant correlations between these two variables for either the 4-day (C) 28-day timepoint (D). Open circles in regression plots identify data points from GDX animals, and R^2 values from the regression analyses appear in the upper left.

Chapter III

The distribution of intracellular androgen receptor immunoreactivity among neurons projecting to the ventral tegmental area in adult male rats

The experiments in the previous chapters of this dissertation expanded evidence for gonadectomy (GDX)-induced impairment of performance on dopamine (DA)-dependent prefrontal cortical (PFC) behavioral tasks (Aubele et al., 2008), and brought to light new evidence that GDX also induces a nearly two-fold increase in basal/resting extracellular PFC DA levels (Aubele and Kritzer, 2010)—all in a manner that is attenuated by replacement with androgen, but not estrogen. Given such strong clues suggesting that observed effects on behavior may be due to a GDX-induced DA dysregulation in the PFC, these results lead to the next obvious questions regarding the site(s) of action for these striking, functionally relevant, androgen-mediated effects on PFC DA systems. Although there are likely to be multiple ways in which androgens impact mesocortical DA systems, mechanistically, all can be presumed to be genomic in nature and translated via intracellular androgen receptors (AR), since androgen actions are only known to occur through this single intracellular receptor isoform. It then follows that one method of revealing potential mechanisms for hormone actions on PFC DA begins by identifying the localization of these ARs within the participating brain circuitry. Given that the major source of PFC DA comes from neurons residing in the ventral tegmental area (VTA, Fallon and Moore 1978), one

explanation for hormone influence over PFC DA systems would be that androgen actions target the cell bodies of origin of the mesoprefrontal DAergic projections located in the VTA. However, previous tract-tracing studies from this lab have shown that only 25% PFC-projecting DAergic cells in the VTA are also AR-immunoreactive (AR-IR, Kritzer and Creutz, 2008) in adult male rats.

Given the substantial effects on DA-dependent PFC behavior and extracellular DA level described in previous chapters of this dissertation, it stands to reason that there may be additional sites of influence in the brain that impact PFC/DA connections. Therefore, the studies in this chapter explored the potential for androgen sensitivity one synapse removed from the VTA. Specifically, the tracer cholera toxin (β subunit) conjugated to fluoro-gold was injected into the VTA and the resultant retrogradely labeled neurons were assessed in combination with immunocytochemistry for intracellular AR to ask whether any of the brain areas afferent to the VTA are also potential sites for androgren regulation, i.e. which neurons projecting to the VTA were also AR-IR. It must be borne in mind, however, that in injecting the VTA, where there is significant intermingling of DAergic that project to PFC, motor, premotor, and to mesolimbic structures including the nucleus accumbens and the amygdala necessarily make our investigation inclusive of and not discriminating among afferents affecting mesocortical, mesoprefrontal and mesolimbic DA systems (Fallon et al., 1978; Fallon and Moore, 1978; Fallon, 1981; Fallon and

Loughlin, 1982; Fallon, 1988). While the physiology and function of both the mesocortical and mesolimbic DAergic systems are well-known to be sensitive to androgens in males (Caggiula et al., 1973; Caggiula et al., 1976; Alderson and Baum, 1981; Mitchell and Stewart, 1989; Bitar et al., 1991; Kritzer, 1997), it was nevertheless the goal of this study to focus on mesocortical DAergic systems to the extent possible. Thus, injections were small and confined to the rostroventral/lateral corner of this area, as these sectors of the VTA have been previously shown to be more enriched with PFC-projecting DAergic neurons (Lammel et al., 2008). The discussion of the data that were obtained likewise will emphasize the mesocortical DAergic system over DA systems eminating from the VTA.

Methods

Animal Subjects: A total of 5 gonadally intact adult male Sprague Dawley rats were used (200–300 g, Taconic Farms, Germantown, NY). Each animal received an injection of the retrograde tracer cholera toxin (β subunit, conjugated to 7 nm colloidal gold, List Biologicals, Campbell, CA), in the ventral tegmental area (VTA) 10 days before euthanasia. All procedures minimized animal use and their discomfort, and were approved by the Institutional Animal Care and Use Committee of Stony Brook University.

Surgery: Craniotomies were carried out under aseptic conditions and used and intraperitoneal injection of ketamine (0.9 mg/kg) and xylazine

(0.5 mg/kg) as anesthesia. Once anesthetized, rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA), a midline incision was made to expose the skull, and single burr holes were drilled over the VTA in the left hemisphere guided by coordinates adapted from the atlas of Paxinos and Watson (1998). Calibrated glass micropipettes containing the cholera toxin tracer diluted to 0.025% in sterile water were then lowered to reach the VTA. Once there, a total of ~50 nL of the tracer was pressure-injected in 10-15 nl increments over a 30 min period using visual monitoring of the meniscus to control volume. After injection, pipettes were left in place for an additional 15 min before being slowly withdrawn. Burr holes were then packed with sterile gel foam and incisions were closed using prolene sutures.

Tissue preparation: Following a 10 day post-surgical survival period, animals were transcardially perfused. For this, rats were deeply anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively). After deep reflexes disappeared, animals were transcardially perfused first with phosphate buffered saline, pH 7.4, followed by 800 mL of 0.1 M phosphate buffer (PB), pH 7.4, containing 2% paraformaldehyde and 15% picric acid at a flow rate of 30 mL/min. After perfusion, brains were removed, post-fixed in the same fixative solution for one hour at room temperature, cryoprotected in 30% sucrose in 0.1 M PB, and were rapidly frozen in powdered dry ice before serial sectioning (40 μm) on a freezing microtome.

Histology: Every third section through the ventral midbrain was silver-intensified to visualize the boundaries of injection sites, and separate one-in-six series of sections through the rest of the brain were silver-reacted and nissl-stained or silver-reacted and immunolabeled using anti-intracellular androgen receptor (AR) antibodies.

Silver intensification of colloidal gold: The gold-conjugated cholera toxin tracer was revealed by a silver-plating reaction. For this, free-floating tissue sections were rinsed in 0.1 M PB, pH 7.4 (4 x 15 min each), incubated in IntenSE silver enhancement solution (4 x 30 min each; GE Healthcare Life Sciences), rinsed again in 0.1 M PB, and fixed in 2.5% sodium thiosulfate (10 min). Sections were then rinsed in 0.1 M PB, slide-mounted, counterstained with 0.5% cresyl violet, dehydrated, and placed under coverslips.

Tracer visualization combined with immunocytochemistry: For the double-labeling protocol, sections were first incubated in 1% H_2O_2 in 0.1 M PB (40 min) and then silver enhanced as above. After silver intensification, sections were rinsed in Tris-buffered saline (TBS), pH 7.4 (3 x 15 min each), blocked in 10% normal swine serum (NSS) in TBS for 2 hours at room temperature, and placed in primary antisera (anti-AR, rabbit polyclonal, Millipore, catalog no. 06-680, lot no. 33529; antigen-synthetic peptide, 21 amino acid N-terminal sequence of human ARs, working dilution 1:1000) diluted in TBS containing 1% NSS. After incubation for 4

days (4℃), sections were rinsed in TBS and placed in biotinylated secondary antibodies (Vector Laboratories, Temecula, CA, working dilution1:100) for 2 hours at room temperature. After further rinsing in TBS, sections were incubated in avidin-conjugated horseradish peroxidase solution [ABC (avidin–biotin complex) kit, Vector Laboratories)] for 2 hours at room temperature, rinsed again in TBS and then in TB. The tissue was then reacted using 3,3'-diaminobenzidine (DAB)-nickel solution (0.014% DAB and 0.6% nickel ammonium sulfate in TB, pH 8.0) as chromagen. After the reaction was complete, sections were rinsed in TB, slide-mounted, dehydrated, and placed under coverslips.

Antibody specificity and control experiments: The AR antibody used is commercially available. Its selectivity for paraformaldehyde-fixed rat brain was additionally evaluated in representative sections of adult rat forebrain and hindbrain using the immunolabeling protocols described above, both alone and in combination with silver intensification, performed with omission of primary or secondary antisera.

Case selection: Injection site boundaries were mapped relative to local cytoarchitecture in a 1-in-3 series of silver-intensified, Nissl stained sections through the midbrain. Only those cases where injection sites appeared to be confined to the rostroventral ventral tegmental area (VTA) and where patterns of resultant labeling were similar to those described in previous studies of VTA connections (Geisler and Zahm, Phillipson) were

considered for analysis. However, to be included the cases also had to have injection sites that were well-matched for size, anterior/posterior and dorsal/ventral location across subjects and show immunoreactivity for AR (AR-IR) that had nuclear signal that was readily discernable from background and that showed patterns of localization and apparent densities that were similar to those previously described for adult male rats (Simerly 1990).

Data analysis: All analyses were performed by a single observer (T.A.). For each selected case, the distributions of retrogradely labeled cell populations were assessed within one-in-six series of tissue sections using a Zeiss Axioskop microscope. Low-power objectives (10x) and dark-field optics were used to evaluate regional patterns of retrograde labeling and AR-IR and to localize this single-labeling to cytoarchitectonically identified regions of interest. Higher-power objectives (40x) and bright-field optics were used to assess single- and especially double- labeling within identified regions at the cellular level.

For all regions of interest, uncorrected counts were made of all visible singly (retrograde tracer) and doubly (retrograde tracer + AR-IR) labeled subpopulations of cells within representative fields. Retrogradely-labeled cells were identified by above-background (4x) levels of silver grain throughout their cytoplasm; for inclusion in counting, these cells also had to have visible nuclei. Double-labeled cells were identified as those cells

containing above background silver-grain levels within cytoplasm that surrounded a nucleus that had above-background levels of blue-black DAB-nickel reaction product. The percentages of all includable (visible nucleus) retrogradely labeled cells that were also AR-IR were calculated on per-structure and per-hemisphere bases from within a minimum of three non-overlapping counting frames (257 x 425 µm) positioned over the labeled field; the upper and right-hand sides of these frames were exclusion lines. Every region that contained retrograde labeling was evaluated in this manner, and for the majority of these 100 or more back-labeled cells per animal, per region and per hemisphere were ifound. However, in the olfactory tubercle, medial septum, magnocellular preoptic area, and pedunculopontine nucleus fewer than 30 total back-labeled cells meeting inclusion criteria per animal were present; the data from these very sparsely labeled areas are not reported.

Results

Distribution of Retrograde Labeling and Androgen Receptor Immunoreactivity:

The regional distributions and apparent densities of neurons retrogradely labeled by injection sites confined to the rostroventral VTA were similar in all of the cases evaluated (See Figure III.1). The

distribution of labeling was also comparable to that previously described (Geisler and Zahm, 2005, Phillipson). For example, retrogradely labeled neurons were distributed within cohesive medially to ventromedially located bands that stretched longitudinally from medial pregenual PFC to mid- medullary levels and that intersected but did not necessarily fill a number of brain nuclei/regions. At all levels, labeling was bilateral but was noticeably more dense ipsilateral to injection sites in structures located rostral to them, and more evenly distributed across the two hemispheres in structures located caudal to them (Geisler and Zahm 2005). The finer details of label distribution also conformed to prior descriptions (see Table III.1, Geisler and Zahm 2005). Rostrally, for example, retrograde labeling was most dense in medial prefrontal cortices, the shell of the nucleus accumbens and in the lateral septal and habenular nuclei; and was moderately dense in the claustrum, bed nucleus of the stria termalis, ventral pallidum, lateral preoptic area, lateral hypothalamus, zona incerta and amygdala. One difference from previous studies, however, was that retrograde labeling in all cases assessed here was sparse along midline structures such as the olfactory tubercle, medial preoptic area, magnocellular preoptic area, medial septum and paraventricular nucleus of the thalamus.

The distributions of AR-IR cells were similar in all cases evaluated and were consistent with previous descriptions of the cellular and regional distributions of AR proteins and mRNAs (Simerly et al. 1990) in adult male

rats. Thus, the DAB-nickel reaction product was most concentrated over the nucleus, with some cells additionally showing AR-IR lighter labeling over the cytoplasm as well; this labeling was widespread and particularly abundant in pyramidal cell layers of the cerebral cortex, in limbic areas such as the amygdala, hippocampus, and nucleus accumbens; in hypothalamus, thamalus and epithalamus; and in the periaqueductal grey. Important for this study is that there were moderate to substantial numbers of AR-IR cells in all of the brain regions that were retrogredly labeled by VTA injections. However, as described in the next section, it was only in specific subsets of VTA-projecting fields that there was appreciable overlap of these two labels within single cells.

Quantitative Assessment of the Cellular Coincidence of Retrograde Tracer and Androgen Receptor Immunoreactivity

Although numerous brain areas were retrogradely labeled by VTA injections, many contained few to virtually no cells that were also AR-IR. For example, for every animal subject, despite there being substantial back-labeling and AR-IR present, fewer than 5% of all retrogreadely labeled cells observed in the ipsilateral or contralateral claustrum, nucleus accumbens shell, bed nucleus of the stria terminalis, amygdala, ventral pallidum, zona incerta, superior colliculus, retrorubral fields and substantia nigra were also AR-IR. For the the lateral preoptic area, lateral hypothalamus, medial and lateral habenular nuclei, and the median raphe nucleus the proportions of retrogradely labeled cells that were also AR-IR

were only slightly higher, with doubly-labeled cells in the injected and uninjected hemispheres accounting for only some 6-17% of all back-labeled neurons. Finally, double-labeling was also modest in the lateral septal nucleus. However, despite there being more retrograde labeling ipsilateral to injection sites, the percentage of doubly-labeled cells on the injected side was consistently about 35% lower from case to case than that present on the uninjected side.

More substantial overlap between retrograde tracer and AR immunoreactivity was observed in the periaqueductal grey (21.79%± 1.05%), the dorsal raphe nuclei (24.25%± 1.44%), the lateral dorsal tegmental nucleus (23.11% ± 2.04%) and the locus ceruleus (35.83 ± 2.03%). However, significantly more and indeed the most double-labeling was found in the pregenual medial prefrontal cortex. Here, retrogradely labeled cells were more dense ipsilaterally than contralaterally, were denser in area IL compared to area PL compared to area Acd, and were localized in layer V, with layer VI of areas PL and IL also containing modest numbers of back-labeled cells (FIG). In terms of double-labeling, ipsilateral to the injection sites, more than half of all back-labeled cells present in layer V (Acd- 60.21 ±2.25%; PL- 51.11 ± 2.87%; IL- 52.28 ± 2.96%) and about one third of all back-labeled cells in layer VI (PL- 37.25 ± 2.48%; IL- 33.96 ± 3.00%) were AR-IR, while contralaterally overlap between retrograde labeling and AR-IR in layer V was about 10% lower than that in the injected side (Acd- 49.40 ± 3.43%; PL- 43.56 ± 2.01%; IL-

40.69 ± 2.71%) and similar to the ipsilateral side in contralateral layer VI (PL- 36.03 ± 1.36%; IL- 36.30 ± 1.05%, see Figure III.2).

Discussion

The ventral tegmental area (VTA), also known as Area A10 of Dahlstroem and Fuxe (Dahlstroem and Fuxe, 1964), is a cluster of intermingled dopamine (DA) and non-DA cells that include those giving rise to the mesocortical and mesolimbic pathways which provide the principal DA input to association and motor cortical areas and to limbic areas such as the nucleus accumbens and amygdala, respectively**.** These distinct DA systems are ascribed with complex processes that range from decision making to motivational functions (Goldman-Rakic et al., 1990; Oades, 2008; Koob and Volkow, 2010), many of which have also been shown to be sexually dimorphic and/or sensitive to gonadal hormone stimulation (Caggiula et al., 1976; Mitchell and Stewart, 1989; Creutz and Kritzer, 2004; Hu et al., 2004; Kritzer and Creutz, 2008; Becker, 2009; Duchesne et al., 2009). Further, in males the majority of gonadal steroid effects that have been identified for both of these forebrain DA systems have been specifically identified as androgen-sensitive and estrogen-insensitive (Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008; Becker, 2009). Unlike estrogen stimulation, which is known to occur via a multiplicity of both classical genomic/transcriptional intracellular (including α and β) and membrane-bound (Boulware and

Mermelstein, 2005; Mhyre and Dorsa, 2006; Walf and Frye, 2006) receptor subtypes, androgen actions are currently believed to occur primarily through a single pathway involving a single intracellular androgen receptor (AR) isoform (Evans, 1988). Thus, in considering the central question of this chapter -- where androgens impact mesocortical and mesolimbic DA systems, the localization of AR with respect to these pathways is likely to give a good first approximation of where the relevant spheres of influence lie.

Not surprisingly, the question of AR localization among mesocortical and mesolimbic DA systems in adult male rats has already been asked, answered, and to a degree eliminated for the DAergic cells of origin of these two pathways in the VTA, where at most 25% and more often only some 4-10% of DA cells projecting to the PFC, amygdala, and nucleus accumbens core and shell have been shown to be AR-IR (Creutz and Kritzer, 2004; Kritzer and Creutz, 2008). Here, however, the same strategy of co-identifying cells by their projections and by their AR phenotypes has identified several discrete populations of neurons that are afferent to the VTA that appear to be substantially more sensitive to androgen stimulation than the DAergic VTA neurons themselves. Thus, while many neuronal pools projecting to the VTA had little to no AR immunoreactivity, in the sections below the dorsal raphe nuclei, locus ceruleus and lateral dorsal tegmental nucleus– which all had more than 20% of constituent cells that were also AR-IR are considered in terms of

their potential contributions to hormone sensitivity of VTA DA systems. Separate consideration is also given to the medial PFC where remarkably more than 50% of retrogradely labeled cells were AR-IR. First, however the pattern of retrograde labeling that was observed in this study is compared and contrasted with that described in previous studies of afferent projections to the VTA.

Retrograde Labeling of VTA Afferents: Comparison to Previous Studies.

The distributions of retrogradely labeled cells produced by injections made in this study in the rostroventral/lateral VTA were largely concordant with those described in previous studies of afferent projections to the VTA using fluorescent or horseradish-peroxidase conjugated tracers (Phillipson, 1978; Geisler and Zahm, 2005). However, there were some discrepancies noted between the labeling observed here and that seen previously, particularly along the midline. In this study, for example, no back-labeled cells were seen in the medial preoptic area and medial septum, and only sparse labeling was seen in other medially-situated areas such as the supramammilary nucleus, tuber cinereum, dorsal periacqueductal grey and median raphe. This contrasts with the moderate to dense labeling reported in previous studies for all of these areas (Phillipson, 1978; Geisler and Zahm, 2005). These differences may be explained, however, by the confinement of injection sites used here to the rostroventral/lateral VTA. In trying to place injection sites in portions of the

VTA that are relatively enriched with mesocortical cells of origin, the strategy in this study was to deliberately use small, more circumscribed injection sites that were located within this particular subfield of the VTA. Given this, the resultant paucity of medially located retrograde labeling in our studies might be predicted by VTA topography. It is known, for example, that medial portions of the VTA project to medially located structures (Fallon and Moore, 1978), and it is possible that reciprocal projections are similarly organized. Thus, while the differences between patterns of labeling described here versus previously could also be explained by differences in the size and spread of the tracer injection site and the different tracer substances used, labeling differences may be a consequence of study to study differences in precise injection site locations.

Neurochemically Diverse Projections as Potential Sites of Androgen Influence over VTA Dopamine Systems.

This study revealed a small number of projections to the VTA as probable sites for androgren stimulation that could be relevant for the numerous instances of hormone modulation/ GDX effects on mesolimbic and mesocortical DA systems that have been described in adult male rats (Caggiula et al., 1976; Mitchell and Stewart, 1989; Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008; Becker, 2009). Interestingly, these include connections from the locus ceruleus, dorsal

raphe and lateral dorsal tegmental nucleus which are likely to be noradrenergic, serotonergic, and cholinergic, respectively. Evidence that selective chemical lesions of norepinephrine neurons in the locus ceruleus and of serotonergic neurons in the dorsal raphe nuclei both increase burst firing of DA neurons in the VTA (Guiard et al., 2008) suggest that these two afferent systems may act to tonically inhibit the VTA and hold its constituent DAergic cells in their quiescent, single-cell spiking rather than their event-related burst-firing modes (Overton and Clark, 1997). The predominance of asymmetric synaptic contacts made by afferents from the lateral dorsal tegmental nucleus, on the other hand (Omelchenko and Sesack, 2005) suggest an excitatory, presumed nicotinic cholinergic drive arising from this structure that could facilitate or support burst firing among VTA DAergic cells. Pharmacologically, each of these three neurotransmitter systems have also been repeatedly implicated in the pathphysiology and or/ treatment of disorders including addiction, depression, ADHD and schizophrenia, which are each well known for links to the mesocortical and mesolimbic DA systems (Viggiano et al., 2003; Hains and Arnsten, 2008; Oades, 2008; Arnsten, 2009; Del Arco and Mora, 2009; Dremencov et al., 2009; Scarr and Dean, 2009). As there are both sex differences in the occurrence and treatments of these disorders and etiological hypotheses linking interactive actions of norepinephrine, acetylcholine, serotonin, and DA to their pathophysiological bases (Halbreich and Lumley, 1993; Dursun and Kutcher, 1999; Piccinelli and

Wilkinson, 2000; Salin-Pascual et al., 2003; Solomon and Herman, 2009), there is strong impetus for future studies to verify the transmitter phenotypes of AR-IR cells within these pathways and to begin to assess hormone impact at the level of interactions between these converging neurotransmitter systems/projectionsm especially in males.

Glutamatergic Projections from the PFC as Potential Sites of Androgren Influence over VTA Dopamine Systems.

By far the most substantial populations of potentially androgen-sensitive VTA projection neurons were found in the medial PFC. These infragranular PFC pyramidal cells are key parts of complex regulatory pathways involving looped corticofugal connections between the PFC, VTA, and nucleus accumbens. It is well-known, for example, that these PFC projections make disynaptic connections from VTA DAergic cells to the nucleus accumbens, and monosynaptic contacts on GABAergic cells in the VTA that project to the mesolimbic nucleus accumbens and/or DAergic VTA cells that project back to the PFC in a short, closed loop circuit (Carr et al., 1999; Carr and Sesack, 2000). Among these mPFC afferents, it is impossible to know which of the 50% of androgen-bearing cells identified in this chapter make synapses on which target cells. For example, is there a potentially higher fidelity to mesoprefrontally-projecting DAergic cells in the VTA than to other cell types in the VTA? Such questions, however, are technically challenging and require at a minimum

the application of trans-neuronal tracers, e.g. viruses or ^{3}H amino acids combined with electron microscopy and/or triple-label immunocytochemistry (Card et al., 1993; Ugolini, 2010). This is an avenue for future study. However, in the meantime, unlike the projections from the locus ceruleus, dorsal raphe nuclei, and lateral dorsal tegmental nucleus above, there is no question about the neurochemical identity of the AR-IR PFC cells as glutamatergic. In the final chapter of this thesis, the hypothesis that these anatomical findings suggest --that glutamate systems may serve as requisite translators for androgen's influence on PFC DA systems -- is directly tested using *in vivo* microdialysis combined with glutamate receptor subtype-selective drug challenge.

Retrogradely Labeled Area	Ipsilateral	Contralateral
Medial Prefrontal Cortex	+++++	++++
Claustrum	+++	++
Olfactory Tubercle	+	-
Nucleus Accumbens Shell	++++	++
Bed Nucleus of Stria Terminalis	++++	++
Amygdala	++	+
Ventral Pallidum	++++	+++
Lateral Septal Nuclei	+++	++
Median Preoptic Area	-	-
Medial Preoptic Area	+	-
Lateral Preoptic Area	+++	++
Magnocellular Preoptic Area	+	-
Tuber Cinereum	+	-
Lateral Hypothalamus	+++++	+++
Supramammillary Nucelus	+	-
Zona Incerta	++	+
Medial Habenula	++++	+++
Lateral Habenula	+++++	++++
Superior Colliculus	++	+
Periaqueductal Grey	+++	+++
Retrorubral Fields	+	+
Dorsal Raphe	++++	++++
Median Raphe	++	++
Pedunculopontine Tegmental Nucleus	+	+
Lateraldorsal Tegmental Nucleus	+++	++
Locus Ceruleus	+++	+++

Table III.1. Relative density of retrogradely labeled neurons in brain structures after injection of the retrograde tracer cholera toxin conjugated to Fluoro-Gold into the ventral tegmental area.

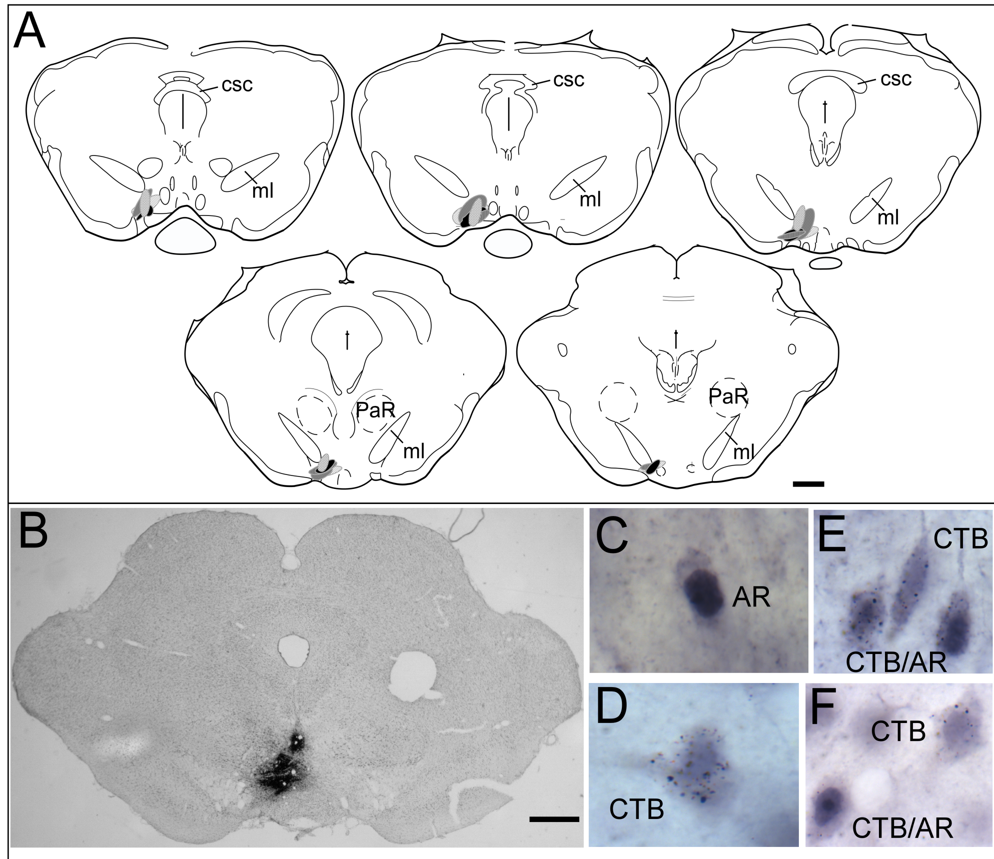

Figure III.1. A. Schematic representations of all injection sites evaluated in the five male subjects of this study, organized rostrocaudally from left to right, top to bottom. Section outlines are adapted from the atlas of Paxinos and Watson (1998). B. Low-power bright-field photomicrograph showing a representative injection site in the VTA. C-D. High-power color photomicrographs showing the appearance of representative neurons retrogradely labeled by cortical injections of cholera toxin (CTB, C) or immunoreactive for intracellular androgen receptor (AR, D). E-F. High-power color photomicrographs showing adjacent cells either singly (CTB) or doubly (CTB/AR) labeled by these markers. Retrograde labeling is identified by silver-enhanced gold particle grains in the cell somata and proximal processes and hormone receptor immunoreactivity appears as blue-black reaction product over cell nuclei. Scale bars =1 mm. Additional abbreviations: csc, commissure of superior colliculus; ml, medial lemniscus; PaR, pararubral nucleus.

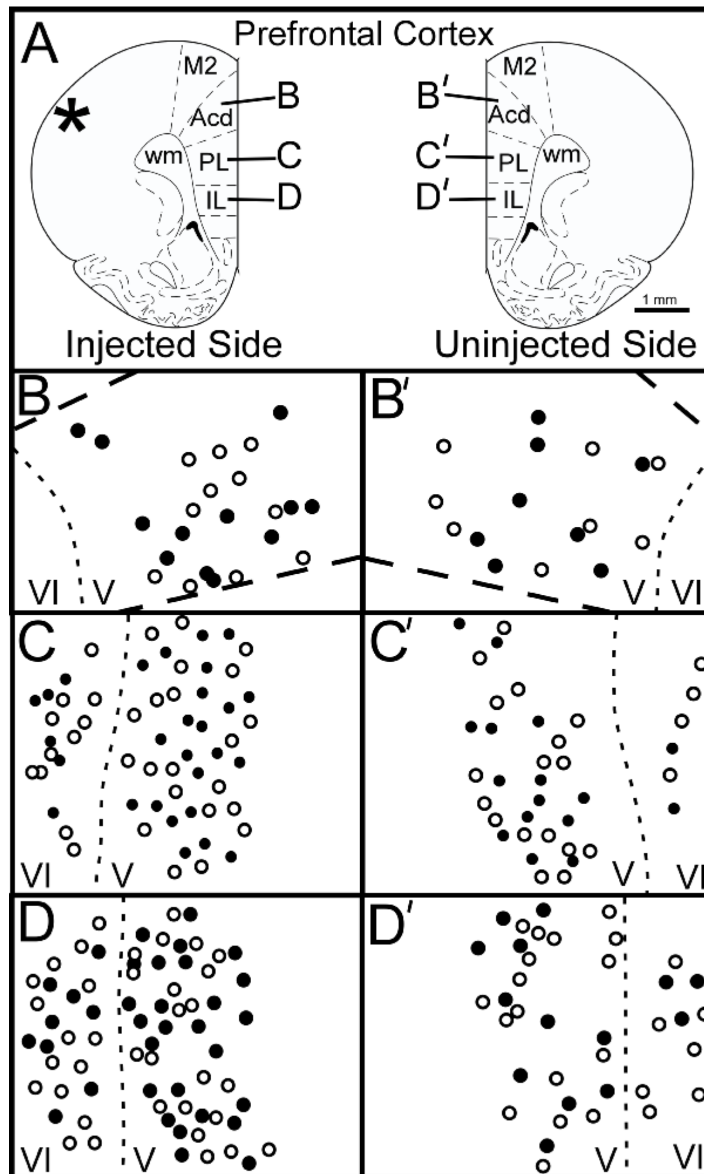

Figure III.2. Plots showing the location of medial prefrontal cortical neurons retrogradely labeled by cholera toxin injection of the ventral tegmental area. Labeling on the side of the PFC ipsilateral to the injection site (*) is shown separately from that seen contralateral to the injection site (A). Anatomical boundaries and labeled cells were mapped using a camera lucida system. Symbols identify each plotted cell as either singly labeled with retrograde tracer (white circles) or double-labeled for retrograde tracer and androgen receptor (AR) immunoreactivty (AR-IR, black circles).Labeling in sections from injected (Ipsi.; B, C, D) and uninjected hemispheres (B`, C`, D`) are shown. While both the density of retrograde labeling and overlap between retrograde labeling and AR-IR was higher in the injected hemisphere, significant proportions of retrogradely labeled neurons in both hemispheres were AR-IR. Roman numerals (V, VI) in the lower right-hand corner of panels indicate cortical layers. Additional abbreviations: M2, premotor cortex; Cg1, anterior cingulate cortex; Prl, Prelimbic cortex; IL, infralimbic cortex; wm, white matter.

Chapter IV

Effects of gonadectomy and hormone replacement on glutamate-stimulated extracellular dopamine levels in prefrontal cortex in adult male rats

The prefrontal cortices (PFC) have been repeatedly implicated in some of the most debilitating forms of cognitive dysfunction known in mental illness (Egan et al., 2001; Goldberg et al., 2003; Winterer and Weinberger, 2004). While it has long been recognized that both the function and the dysfunction of the PFC in disease are strongly influenced by its dopamine (DA) inputs (Tassin et al., 1978; Kessler and Markowitsch, 1981; Kalsbeek et al., 1989; Davis et al., 1991; Murphy et al., 1996; Verma and Moghaddam, 1996; Zahrt et al., 1997; Morrow et al., 2000; Goto and Grace, 2007), it has also become increasingly clear that glutamate also plays critical roles in PFC function and dysfunction-- in large part via its effects on mesoprefrontal DA systems. For example, in rats reverse-dialysis application of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonists not only elevate and depress extracellular PFC DA levels, respectively (Feenstra et al., 1995; Jedema and Moghddam, 1996; Jin, 1997; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Del Arco et al., 2008), but significantly impair performance in classical PFC tasks, including those previously recognized as exquisitely sensitive to either elevated or depressed PFC DA signaling (Stefani et al., 2003; Stefani and

Moghaddam, 2005). Such integrated influences are also at the heart of contemporary hypotheses regarding the prefrontal deficit/cognitive symptoms of schizophrenia, which have evolved from purely DA-based theories (Langer et al., 1981; Abi-Dargham and Moore, 2003; Abi-Dargham, 2004; Murray et al., 2008; Howes and Kapur, 2009) to etiological arguments that posit dysregulation at levels of DA/glutamate interactions (Moghaddam, 2003; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010). Stimulated by evidence of hormone sensitivity and of male over female sexual dimorphisms in both PFC function and its dysfunction in disease (Dawson et al., 1975; Einon, 1980; Seeman and Lang, 1990; Leviton et al., 1993; Wickelgren, 1997; Overman, 2004; Luine, 2008; Elsabagh et al., 2009), this lab has previously used an adult male rat model to explore the effects of gonadal steroids on the PFC's pivotal DA innervation in this sex. However, recognizing that not only DA but glutamate/DA interactions are critical to PFC operations, the present study now uses this same animal model to assess the hormone sensitivity of the cortical interactions between these amine and amino acid neurotransmitters.

There is considerable evidence for gonadal hormone stimulation/sensitivity of PFC function in males. In adult men, for example, high levels of circulating testosterone are correlated with superior performance in cognitive tasks such as mental rotation (Young et al., 2010), route-learning (Cherrier et al., 2001; Cherrier et al., 2007) and word

recall (Cherrier et al., 2001; Cherrier et al., 2007) while the low hormone titers that accompany aging, androgen deprivation therapies and Klinefelter's syndrome -- the most common sex chromosome disorder (Bojesen et al., 2003; Morris et al., 2008) are associated with reduced verbal fluency and spatial working memory (Patwardhan et al., 2000; Cherrier et al., 2001; Janowsky, 2006; Cherrier et al., 2007; Nelson et al., 2007). In schizophrenic men low circulating testosterone levels have also been correlated with the severity of the cognitive/PFC symptoms associated with this disorder (Shirayama et al., 2002; Goyal et al., 2004; Taherianfard and Shariaty, 2004; Akhondzadeh et al., 2006; Janowsky, 2006; Ko et al., 2007; Nelson et al., 2007). To understand both the positive and negative impact that gonadal steroid hormones have on PFC operations in males, this lab and others have used an adult male rat model to explore how hormones affect essential PFC DA systems. However, while these studies have identified significant effects of GDX and hormone replacement on, for example, DA axon density (Kritzer et al., 1999; Kritzer, 2000; Kritzer and Pugach, 2001), PFC DA uptake and reuptake (Meyers and Kritzer, 2009; Meyers et al., 2010) and on the acquisition (Ceccarelli et al., 2001; Kritzer et al., 2001; Daniel et al., 2003; Kritzer et al., 2007) and performance (Adler et al., 1999; Sandstrom et al., 2006; Aubele et al., 2008; Gibbs and Johnson, 2008) of DA-dependent PFC tasks, questions of hormone sensitivity of PFC glutamate/DA interactions have never been explored. To begin to address this issue, the

studies in this chapter paired long-term GDX and hormone replacement with *in vivo* microdialysis and reverse-dialysis application of selective NMDA and AMPA antagonists. While previous studies have shown that this GDX model induces a nearly 2-fold increase in resting/basal extracellular DA levels in the PFC (Aubele and Kritzer 2010), here we ask whether GDX and/or hormone replacement with testosterone propionate or estradiol also influence the well-documented abilities of intracortical application of glutamate receptor subtype-selective antagonists to increase or decrease PFC DA levels in predictable ways (Feenstra et al., 1995; Jedema and Moghddam, 1996; Jin, 1997; Takahata and Moghaddam, 1998; Feenstra et al., 2002; Del Arco et al., 2008).

Methods

Animal Subjects: A total of 38 adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were used. Animals were housed in pairs of like treatment under a 12/12 hour light/dark cycle with food and water available *ad libitum.* Ten animals were sham-operated (CTRL); 5 were used in experiments where the NMDA antagonist D (–)-2-Amino-5-phosphonopentanoic acid (APV, below) was administered, and five were used in experiments evaluating the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, below). The remaining 28 animals were gonadectomized (GDX); of these, 5 made up the APV GDX cohort, and 5 comprised the NBQX GDX group. Ten GDX

rats were supplemented with testosterone propionate (TP); of these, 5 were used for the APV GDX-TP cohort, and 5 made up the NBQX GDX-TP group. Eight GDX animals were supplemented with 17-β-estradiol (E); 4 of these rats were used as the APV GDX-E cohort and 4 comprised the NBQX GDX-E group. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize their use and discomfort.

Surgeries: All surgeries were carried out under aseptic conditions and used intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. Rats were monitored during recovery from surgery and given 0.03 mg/kg buprenorphine to manage post-operative discomfort.

Sham surgery or gonadectomy was performed 28 days before microdialysis. For both procedures, the sac of the scrotum and the underlying layers of tunica were incised. For GDX, the vas deferens was also bilaterally ligated and the testes removed. Incisions were closed using sterile wound clips that were removed two weeks post-surgery.

Craniotomy was performed for placement of microdialysis probe guide cannulae on the day before the microdialysis experiment. For this procedure, anesthetized rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA), an incision was made to expose the skull and burr holes were drilled for inserting and anchoring guide cannulae (CMA Microdialysis, North Chelmsford, MA, USA) to the skull. Using coordinates

adapted from the atlas of Paxinos and Watson (1998), cannulae were aimed toward the left pregenual medial prefrontal cortex 3.2 mm anterior to Bregma, and were secured with a combination of shallow screws and dental cement.

Hormone Supplements: For the hormone-supplemented groups, pellets that released either 3–4 ng of TP (GDX-TP rats) or 25 pg of E per milliliter of blood per day (GDX-E rats, Innovative Research of America, Sarasota, FL) were inserted within the tunica at the time of GDX. Both pellet types and their doses have been used in previous investigations in this laboratory and others and have been shown to yield circulating level of gonadal hormones that fall within physiological range (Collins et al., 1992; Adler et al., 1999; Kritzer, 2000).

In Vivo Microdialysis:

Baseline: On the morning after craniotomy, awake animals were placed in Raturn clear rodent bowls (BioAnalytical Systems, West Lafayette, IN, USA) and allowed to acclimate to the environment for 10 minutes before microdialysis probes (100,000 Dalton cut-off, 3mm PES exposed membrane tip, CMA Microdialysis, North Chelmsford, MA, USA) were slowly lowered through guide cannulae into place. For the next 2 hours, the probes were perfused with artificial CSF (145 mM 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 H2O, pH 6.8) at a rate of 2 μL/min for equilibration.

Afterward, dialysates were collected every 20 minutes. This phase of the experiment took place between 0900-1800 hours, which corresponded to the rats' subjective night. At least 3 baseline samples were collected from each animal before drug delivery (below) was initiated; only those samples collected while the animal was and had been asleep for the preceding 20 minutes were used in the analysis.

Drug Challenge: After three stable baseline measurements were obtained, either 500 μM APV (Sigma Chemical Co., St. Louis, MO, U.S.A) or 150 μM NBQX (Sigma Chemical Co.) was added to the artificial CSF (as per above) and delivered via reverse dialysis to the PFC for the next 120 minutes. Behavioral and neurochemical responses to drug administration were evaluated during and after the 2 hour drug delivery periods and until PFC DA levels returned to pre-drug baseline values for at least two successive samples. All testing was complete prior to the onset of animals’ subjective day (1900 hrs).

Dialysis Analysis: All dialysate samples were directly injected into an HPLC system (PM 92-E pump, BAS, West Lafayette, IN) via an on-line autoinjector (Pollen-8, BAS, West Lafayette, IN). Analyses utilized a microbore column (UniJet, 1.0 mm inner diameter, 150mm length, 5 μm ODS particles; BAS, West Lafayette, IN, USA) and a BioAnalytical Systems LC-Epsilon detector (BAS, West Lafayette, IN, USA); the E_{app} was + 0.65 V versus the Ag/AgCl reference electrode and 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; .027 mM EDTA; 7.2% acetonitrile (v/v), 1% tetrahydrofuran (v/v), pH 3.4. Probe efficiency was determined to be 10-18% for all studies, and an overall detection limit of 8 fmol was achieved. All chemicals used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Euthanasia and Histology: At the conclusion of the microdialysis study, animals were euthanized by rapid decapitation and their brains were removed and immersed in a 30% sucrose, 10% buffered formaldehyde solution for fixation and cryoprotection. The androgen-sensitive medial ventral and lateral bulbospongiosus muscles (BSM) were also dissected out and weighed. After the brains sank in the sucrose solution, the frontal cortex region of each subject was blocked, rapidly frozen and serially sectioned in the coronal plane on a freezing microtome at a thickness 40 µm. For each animal subject, a 1 in 4 series of sections taken from mid olfactory bulb through the genu of the corpus callosum was collected, slide mounted, Nissl stained and examined to verify the cytoarchitectonic location of the microdialysis probe. In order for data from a given animal to be included in the analyses, probes had to have been placed in the left pregenual medial PFC, extend dorsoventrally through prelimbic and infralimbic areas but not beyond to dorsopeduncular cortex, and be centered over middle and deep cortical layers (see Figures 1 and 3).

Data Analysis: Dopamine peak identity was confirmed and quantified (ng/10 mL) in relation to standard peaks of known concentrations (1, 2, 5,

10 ng/10mL) run through the HPLC system on the same day as the animal data were collected; uncorrected peak values were measured automatically by ChromGraph software (BAS, West Lafayette, IN, USA) as peak height and were converted to concentrations (fmol/µL) using the standards. Because DA metabolites such as homovanillic acid and 3, 4 dihydroxyphenlyacetic acid were detected in only a subset of the animals, these were not assessed. Pre- and post-drug DA baselines were similarly defined as the averages from at least 3 stable measurements per subject, and drug-induced changes in DA levels were assessed as percents of these averaged baselines and as absolute DA concentrations (fmol/uL). Values from individuals were then compiled and assessed in two ways: within groups analyses using descriptive statistics followed by one-way analyses of variance (ANOVA) to probe for significant main effects of time (drug effect) on DA level, and across groups analyses using descriptive statistics followed by one-way ANOVAs to assess main effects of hormone treatment on drug-induced changes in PFC DA level. Allowed post-hoc testing used the Fisher's Protected Least Significant Difference (PLSD), and in all cases $p < 0.05$ was accepted as significant. BSM weights were also compared using across groups analyses using descriptive statistics followed by one-way ANOVAs. Regression analyses in which the BSM weights were used as independent variables and the individual animals' basal extracellular PFC DA (above) were used as dependent variables were also carried out.

Results

<u>NMDA Receptor Antagonism: APV</u>

The effects of local, reverse dialysis application of the NMDA antagonist APV on extracellular DA levels in the PFC were assessed in rats that were sham operated (CTRL), gonadectomized (GDX) or GDX and supplemented with testosterone propionate (GDX-TP) or estradiol (GDX-E) 28 days prior to microdialysis. The effectiveness of hormone treatments in these animals were confirmed in analyses of the weights of their androgen-sensitive bulbospongiosus muscles (BSM). As expected, these assessments showed that mean muscle weights of the CTRL and GDX-TP groups were similar to each other and some 4-fold higher than those of the GDX and GDX-E rats. Analyses of variance comparing individual animal BSM weights further revealed significant main effects of hormone treatment [$F_{(3,15)} = 9.815$, $p = .0008$] and allowed post-hoc comparisons identified the BSM weights of the CTRL and GDX-TP cohorts as similar and not significantly different from one another, the weights of the GDX and GDX-E rats as similar and not significantly different from each other, and the BSM weights of the GDX and GDX-E cohorts as significantly different from those of both the CTRL and GDX-TP groups (Figure IV.1).

For microdialysis, animals were assessed during their subjective night and were given a two-hour period for habituation and probe equilibration during which time all subjects fell asleep. Afterwards,

baseline samples were collected which yielded group-specific extracellular PFC DA levels in the CTRL and hormone-treated cohorts that were similar to those previously reported (Moghaddam and Jackson, 2004; Stefani and Moghaddam, 2005; Del Arco et al., 2007; van der Meulen et al., 2007; Balla et al., 2009). Thus, extracellular PFC DA levels in CTRL rats ranged from 0.10 - 0.28 fmol/μL (mean = 0.17 ± 0.04 fmol/μL), were roughly twice as high as CTRL in GDX and GDX-E rats (GDX mean = 0.33 ± 0.06 fmol/μL, GDX-E mean = 0.27 ± 0.05 fmol/μL), and were similar to CTRL in the GDX-TP group (mean = 0.12 ± 0.02 fmol/μL, Figure IV.1). Analyses of variance performed on these data identified significant main effects of hormone treatment [$F_{(3,15)} = 339.654$, $p < .0001$], and post-hoc comparisons confirmed that pre-drug baseline DA levels in the GDX and GDX-E but not the GDX-TP group were significantly higher than control (CTRL vs. GDX, $p<.0001$; CTRL vs. GDX-E; $p<.0001$): Regression analyses assessing individual animals' extracellular PFC DA levels as a function of BSM weight also identified significant correlations between mean and individual baseline PFC DA levels and androgen-sensitive muscle weight (individual DA measures: $R^2 = 0.264$, $p < 0.001$ average DA measures: $R^2 = 0.289$, $p < 0.001$, Figure IV.1).

For drug infusion, the perfusate was switched to artificial CSF containing 500 μM of the selective NMDAR antagonist APV. Forty minutes after infusion began, an expected set of mild behavioral responses emerged in the CTRL rats, i.e., the animals awoke and remained largely

stationary but intermittently displayed grooming, stereotyped orofacial/chewing movements and whisking (Feenstra et al., 2002). These behaviors persisted for the remainder of the drug application period and disappeared 40-60 minutes after drug offset. Similar behaviors were also elicited by drug delivery in all three hormone treatment groups. However, in the GDX and GDX-E rats, arousal onset was some 20 minutes sooner and offset some 40 minutes prior to that seen in the CTRL and GDX-TP groups (Figure IV.2A).

Effects of APV infusion on PFC DA levels in CTRL animals were similar to those previously reported for male rats using the same drug, dose, and application schedule (Takahata and Moghaddam, 1998; Feenstra et al., 2002) Thus, within 20 minutes of drug application, extracellular PFC DA levels rose by about 20% and by 60 minutes rose further to plateau at levels of about 40% above pre-drug baseline which were sustained for the duration of the drug application period. Afterward, DA levels began to decrease steadily and returned to pre-drug baseline levels within 40-60 minutes of drug cessation. The drug responses observed in GDX-TP rats were similar to CTRL, although peak effects of APV were about 15% higher and took approximately 60 minutes longer to return to pre-drug baseline levels than in the sham-operated group. In contrast, within 20 minutes of drug infusion, DA levels in both the GDX and GDX-E groups decreased by about 20%, and over the next 20 minutes DA levels fell further to become maximally depressed at about

40% below baseline. These depressed levels persisted for the remainder of the drug application period, after which DA levels rose slowly and returned to pre-drug baseline 80-100 minutes after drug was removed. Quantitative, within-group analyses of these data (one-way ANOVAs) revealed significant or near-significant main effects of time on PFC DA levels [F = 5.934-20.076, p = .0906-.0022] and post-hoc analyses confirmed that for all groups DA levels were similar in pre- and post-drug baseline periods and significantly different ($p<0.05$) from baseline during drug application (Figure IV.2). Across-group ANOVAs also identified significant main effects of hormone treatment on APV-induced changes in extracellular PFC DA levels that began 20 minutes after drug initiation and persisted until 60 minutes after drug cessation [F = 7.664-25.076, p = .0403-.0007]. Allowed post-hoc comparisons further confirmed that APV-induced changes in extracellular DA in CTRL and GDX-TP were similar to each other but significantly different from the GDX and GDX-E groups (all $p<0.05$, Figure IV. 2B) during drug infusion, that GDX, GDX-E and GDX-TP rats all differed from CTRL 20 minutes after drug offset, and that DA levels in the GDX-TP cohort were significantly different from all other groups for the next 40 minutes (all $p<0.05$, Figure IV.2B).

AMPA Receptor Antagonism: NBQX.

An independent set of CTRL, GDX, GDX-TP and GDX-E rats were used for studies using reverse dialysis application of the AMPA antagonist NBQX. As for APV animals, the effectiveness of hormone treatments in

NBQX-treated rats was confirmed in quantitative and statistical analyses of group differences in BSM weights. Expected, significantly significant group differences in baseline measures of extracellular PFC DA were also seen, and regression analyses assessing both individual and group mean extracellular PFC DA levels as a function of BSM weight further showed significant correlations between these two variables (Figure IV.3).

Following collection of baseline dialysates, probes were perfused with artificial CSF containing 150 µM of the selective AMPA antagonist NBQX (2 µL/min, 120 minutes). Unlike APV, the infusion of this drug elicited no obvious behavioral responses in any of the animal groups and all subjects remained asleep for the duration of the experiment. However, during this period there were clear effects in the CTRL and GDX-TP rats on extracellular PFC DA levels. Similar to what has been reported in studies using other selective AMPA receptor antagonists (Takahata and Moghaddam, 1998) within 40-60 minutes of drug onset DA levels in CTRL rats dropped by roughly 30-40% and remained at these depressed values for the duration of the drug application period. After, DA levels rose incrementally to return to pre-drug baseline levels within 80 minutes of drug offset. In the GDX-TP rats, a similar DA-depressing drug response was observed, although in these animals the induced decrease from baseline began and ended 20 than in CTRL. In contrast, there were no appreciable responses to NBQX administration in either the GDX or the GDX-E groups; for both, PFC DA levels remained within 10% of pre-drug

baseline values for entirety of the drug application and post application periods. Statistical analyses of these data supported all of these observations. Thus, within group one-way ANOVAs revealed significant main effects of time on PFC DA levels in the CTRL and GDX-TP but not the GDX or GDX-E groups [$F = 13.961\text{-}19.093$, $p = .0090\text{-}.0132$] and post-hoc comparisons confirmed that only in the former 2 groups as well were DA levels similar in pre- and post-drug periods and significantly different ($p<0.05$) from baseline levels during drug application. Between-group ANOVAs also identified significant main effects of hormone treatment on drug-induced changes in PFC DA levels that emerged 80 minutes after drug application and persisted until drug offset. Allowed post-hoc comparisons further confirmed that the CTRL and GDX-TP groups had significantly decreased extracellular DA levels compared to those of the GDX and GDX-E groups from 80-120 minutes post-drug application (all $p<0.05$, see Figure IV.4).

Discussion

A longstanding interest in the neurobiological basis for hormone impact on the executive functions of the PFC and their disproportionate and disproportionately severe dysfunction in males in schizophrenia and other disorders has led this lab and others to focus on DA as an endpoint of gonadal hormone stimulation in male animal models. However, given the complex receptor subtype-selective control that glutamate exerts over PFC DA levels and guided by current clinically-related theories of PFC

dysfunction that center on integrated intracortical amine and amino acid neurotransmitter actions (Moghaddam, 2003; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010), the present study used reverse dialysis application of the NMDA antagonist APV and the AMPA antagonist NBQX to extend previous analyses of the effects of chronic GDX and hormone replacement on resting/basal levels of extracellular PFC DA levels (Aubele and Kritzer, 2010) to analyses of glutamate-stimulated DA levels in the same GDX animal model. What was discovered was that in CTRL and GDX-TP rats intracortical application of APV induced expected increases in behavioral arousal and extracellular PFC DA level (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998; Feenstra et al., 2002) and that NBQX produced a decrease in extracellular PFC DA similar to that observed in previous studies using structurally similar AMPA antagonist drugs (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998, 2000). However, the responses in GDX and GDX-E rats to both challenge drugs were very different. Specifically, in both the GDX and GDX-E cohorts, APV produced a significant decrease in extracellular PFC DA whereas NBQX infusion had no appreciable effect on PFC DA levels. These findings suggest that 1) while NMDA receptor-mediated stimulation normally exerts a tonic suppressive effect on PFC DA tone (Moghaddam, 2003), in the GDX and GDX-E but not GDX-TP rats there is instead a tonic, NMDA receptor-mediated stimulation of PFC DA level and 2) in the GDX and GDX-E but

not GDX-TP rats the normally present tonic, AMPA receptor-mediated stimulation of PFC DA tone (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998) is decreased to absent.

That there are thus striking androgen-sensitive, estrogen-insensitive effects of GDX on both resting (Aubele and Kritzer, 2010) and glutamate receptor-stimulated PFC DA levels raises questions about mechanism. Given the decidedly comodulatory actions of both DA and glutamate described in, for example, studies of NMDA, AMPA, and D_1 receptor trafficking in cultured PFC neurons (Scott et al., 2002; Sun et al., 2005; Aperia and Greengard, 2006; Gao and Wolf, 2008; Sun et al., 2008; Scott and Aperia, 2009) and in influencing excitability in pyramidal and non-pyramidal PFC neurons *in vivo* and *in vitro* (Cepeda et al., 1999; Wang and O'Donnell, 2001; O'Donnell, 2003; Wang et al., 2003; Tseng and O'Donnell, 2004; Kruse et al., 2009), the question of cause and effect in particular also comes to the fore. Thus, the sections that follow separately discuss whether GDX perhaps induces tonically increased extracellular DA levels which in turn trigger changes in glutamatergic signaling, and whether GDX might rather affect PFC glutamatergic systems which subsequently result in a sustained hyperdopaminergic PFC state. These findings are also related to the limited data available describing hormone actions on neo- and archicortical glutamate systems. These sections are preceded, however, by discussion pertaining to the selection of NBQX as a selective AMPA antagonist and some caveats for

its use in our study-design.

Technical Note: Among available AMPA/kainate selective antagonist drugs, recent evidence has shown that several quinoxalinedione compounds such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-Dinitroquinoxaline-2,3-dione (DNQX) also act as partial glutamate agonists in the presence of transmembrane AMPA receptor regulatory proteins e.g. stargazin, γ3, γ4, γ8 (Tomita et al., 2003; Menuz et al., 2007; Sager et al., 2009) which are known to be present and thus potentially confounding in the rodent cerebral cortex (Tomita et al., 2003). For this reason, NBQX, which does not share these partial-agonist properties (Menuz et al., 2007) was selected for use in this study. This selection, however, came at a cost in terms of solubility. Specifically, while NBQX is soluble in water, it is less so in artificial CSF and proved incompatible with our use of an online autoinjector for HPLC-EC of dialysates at an initially tried and more commonly used concentration of 300 μM (Consolo et al., 1996; Sakai et al., 1997; Giovannini et al., 1998). Although solubility issues were obviated at the lower dose of 150 μM, given the dose-response curves previously described for reverse-dialyses application of CNQX and DNQX in altering subcortical extracellular DA levels and DA-dependent behaviors (Taepavarapruk et al., 2000; Suto et al., 2009), we are necessarily cautious in interpreting the lack of NBQX effect in GDX and GDX-E rats. Throughout the text, these outcomes are consistently identified as evidence of reduced to absent AMPA receptor-mediated

signaling.

Hormone Effects on Glutamate-Receptor Stimulated PFC DA Level: Primary Actions on Mesoprefrontal DA Systems?

Given the localization of intracellular androgen receptor (AR) immunoreactivity within roughly 25% of mesoprefrontally-projecting midbrain DA neurons (Kritzer and Creutz, 2008) it seems logical that at least some hormone actions relevant for the sustained increase extracellular PFC DA, exaggerated excitatory NMDA receptor-mediated activity and reduced to absent AMPA receptor-mediated signaling seen in GDX and GDX-E rats are levied at the midbrain DA cells themselves. However, information from modeling, electrophysiological and other studies may argue against this. For example, recent studies in dopamine transporter (DAT) knockout (KO) mice have shown that while this genetic deletion elevates PFC DA levels by nearly 4-fold, it also produces a sequelae of physiological events that include a switch in PFC DA signaling from D_1 to D_2 receptor-mediated mechanisms. What follows is a D_2 receptor-mediated attenuation of long-term potentiation (LTP) induction and a reduction in the frequency of NMDA receptor-mediated mini excitatory post-synaptic currents (EPSCs) in layer V PFC pyramidal cells (Xu et al., 2009). As layer V is home to pyramidal cells providing a monosynaptic corticofugal excitatory drive on mesoprefrontal DA neurons (Fallon, 1988; Carr and Sesack, 2000), their reduced activation in the DAT KO mice seems more likely to promote a hypodopaminergic rather than

hyperdopaminergic PFC state. That NMDA receptor-mediated actions in the PFC are also blunted in the DAT KO condition is likewise inconsistent with evidence for their abnormal, tonic overactivation in GDX and GDX-E rats. It could be argued, however, that the DAT KO mice are not good comparators, as the lower, 2-fold increase in PFC DA tone seen in GDX and GDX-E rats may be insufficient to trigger a switch from D_1 to D_2 receptor-mediated mechanisms (Law-Tho et al., 1994; Zheng et al., 1999; Seamans et al., 2001). However, an alternative scenario of increased DA signaling at D_1 sites is unlikely to explain the PFC DA abnormalities induced by GDX. First, while modeling studies have shown that D_1 receptor-mediated mechanisms can potentially cause hyperdopaminergia (the so-called "H mode"), to do so requires D1 activation of more than 60% higher than its optimal level (Tanaka et al., 2006), which has been described as physiologically unlikely (Tanaka, 2008). Further, supranormal D_1 actions within a more reasonable range, such as acute application of 100-500 μM SKF38393, dihydrexydine or A77636 to PFC slices (see Yang and Chen '05) diminish NMDA-mediated EPSCs and decrease PFC pyramidal cell firing rate in rodents and primates (Castro et al., 1999; Gao et al., 2001; Seamans and Yang, 2004; Yang and Chen, 2005; Vijayraghavan et al., 2007). In sum, both supranormal D_2-(above) and D_1–stimulation have net results of decreasing PFC pyramidal cell activity, decreasing NMDA receptor-mediated excitatory drive-- and very likely of decreasing PFC DA levels as well (Seamans and Yang, 2004; Hains and

Arnsten, 2008). None of these outcomes fit with the observations made here in the GDX model, which show that that exaggerated NMDA receptivity accompanies tonically elevated extracellular PFC DA levels.

Hormone Effects on Glutamate-Receptor Stimulated of PFC DA Levels: Primary Actions on Glutamatergic Systems?

While it is difficult to support hypotheses of direct hormone actions on PFC DA systems as accounting for the GDX-induced increase in basal/resting extracellular PFC DA and simultaneous exaggeration in NMDA receptivity, there may be some evidence to suggest primary actions of gonadal steroids target local glutamatergic signaling in the PFC, which in turn produce abnormalities in extracellular DA levels. In fact, hormone actions relevant to resultant elevations in PFC DA level might be further defined as principally affecting NMDA receptor-mediated signaling. In GDX and GDX-E rats, for example, the normally upregulating AMPA receptor-mediated drive (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998) is reduced or absent which is difficult to reconcile with the tonically elevated DA levels seen in both groups (Aubele and Kritzer, 2010). NMDA receptor activation on the other hand is known to excite the PFC's fast-spiking, presumed parvalbumin-containing inhibitory interneurons which suppress firing in the corticofugal cells that excite mesoprefrontal DA afferent neurons in the ventral midbrain and yield a tonic disynaptic inhibitory influence on PFC DA tone (Jackson et al., 2004; Homayoun and Moghaddam, 2007). In the GDX and GDX-E rats,

however, NMDA receptors appear to exert a highly abnormal tonic upregulation of PFC DA level. This could be explained by a GDX-induced decrease in NMDA receptor-mediated activation of fast-spiking PFC interneurons. However, it should be noted that many of these parvalbumin-immunoreactive interneurons are immunoreactive for intracellular estrogen but not androgen receptors (Kritzer, 2002; Kritzer, 2004), which does not immediately gel with the androgen-sensitive and estrogen-insensitive nature of the GDX-induced changes PFC DA level observed (Aubele and Kritzer, 2010). It is also possible, however, that changes wrought by GDX are mediated by an increase in NMDA receptivity among PFC pyramidal cells and specifically those projecting to the ventral midbrain. In considering this possibility, it is interesting to note that more than 60% of PFC pyramidal cells projecting to the VTA have been found to be AR- but not ER-immunoreactive (Kritzer, 2002; Aubele et al. 2009). Thus, the substrates for an otherwise, perhaps unlikely cell-specific pattern of hormone impact that is necessary to explain the androgen-sensitive effects of GDX on NMDA stimulated PFC DA levels appear to be in place. As discussed in the following section, in considering the possibility for GDX-stimulation and androgenic hormone suppression of NMDA receptor-mediated pyramidal cell excitability in the PFC further, some additional support for this scenario comes from previous morphological, electrophysiological and receptor-binding studies carried out in neocortical and archicortical areas in male rats.

Previous Studies: Evidence for Hormone Modulation of Glutamate

As for many neurotransmitter systems, studies that have explored glutamate's hormone sensitivity have focused mainly on reproductive and endocrine brain centers and on female subjects. However, there are a small number of studies that have examined the effects of GDX or treatment with androgenic anabolic steroids in the cerebral cortex of male animal subjects (Brann et al., 1993; Le Greves et al., 1997; de Olmos et al., 2008) that support androgen regulation of NMDA receptor function in the male cerebrum. For example, consistent with our data suggesting a suppressive androgen influence over PFC NMDA receptor-mediated actions are findings that retrosplenial cortical neurons are less sensitive to excitotoxicity induced by the selective NMDA antagonist MK-801 in male compared to female rats (Honack and Loscher, 1993; Fix et al., 1995; D'Souza et al., 2002; Bueno et al., 2003) and in GDX male rats given TP or DHT, but not E compared to GDX alone (de Olmos et al., 2008).

In contrast to cerebral cortex, more studies have examined gonadal hormone effects on receptor-subtype selective glutamate signaling in the archicortex, i.e., the hippocampus, a structure for which there is a particularly large body of evidence showing potent estrus-cycle and estrogen-mediated, NMDA-dependent influences on the excitability, amenability to long-term potentiation (Foy et al., 1999; Nilsen et al., 2002; Smith et al., 2009; Zadran et al., 2009) and dendritic spine density (Murphy and Segal, 1996; Woolley et al., 1997; Woolley, 1998; Smith et

al., 2009) of hippocampal pyramidal neurons in female rats (Gould et al., 1990; Wong and Moss, 1991; Woolley et al., 1997; Woolley, 1998; McEwen and Alves, 1999). A smaller number of studies have also identified corresponding effects for androgen deprivation and replacement in the hippocampus of males. For example, long-term adult and peri-pubertal GDX have been shown to stimulate pyramidal cell excitability, lower threshold for the induction of LTP, and increase ^{125}I MK801-binding in a TP and/or DHT reversible manner (Kus et al., 1995; Handa et al., 1997; Harley et al., 2000) in the CA1 region of hippocampus. These findings are all consistent with observations made in this study which are indicative of enhanced PFC NMDA receptor-mediated function in chronically GDX animals that is reversed by TP but not E. Further, assuming that upregulated extracellular PFC DA levels observed in this study in long-term (28 day) GDX rats are related to increased NMDA receptivity, it might also be that the decreased PFC DA levels seen in short-term (4 day) GDX rats (Aubele and Kritzer 2010) are concomitant with decreased NMDA receptor-mediated signaling. This scenario in fact fits recent evidence showing that short-term (3 or 7 days) GDX diminishes NMDA-displaceable hippocampal ^{3}H-glutamate binding (Romeo et al., 2005) and decreases pyramidal cell spine density (Leranth et al., 2003) in a TP and/or dihydrotestosterone (DHT) reversible manner.

Summary and Conclusions

While important contributions of DA to PFC function and its dysfunction in disease are undeniable, this pivotal transmitter operates within a larger framework that includes essential modulation and co-modulation via other amine neurotransmitters e.g. norepinephrine (NE, Arnsten, 1997; Arnsten and Li, 2005) and the amino acid neurotransmitters GABA and glutamate (Moghaddam, 2003; Sesack et al., 2003; Coyle, 2004; Del Arco and Mora, 2005; Gray and Roth, 2007; Fuxe et al., 2008). In exploring means whereby gonadal hormones exert their profound influence over PFC function and its dysfunction in disease in males, this study thus extended previous assessments of PFC DA systems (Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008) to evaluation of glutamate/DA interactions within the PFC. While it has been previously shown that GDX elevates resting/basal extracellular PFC DA levels by almost 2-fold (Aubele and Kritzer, 2010), this study showed a striking, GDX-induced, androgen-sensitive dysregulation of PFC DA levels stimulated by reverse-dialysis application of glutamate receptor subtype-selective antagonists. The significantly muted responses to NBQX and the virtually opposite responses to APV observed in the GDX and GDX-E compared to GDX-TP and CTRL rats suggests that long-term GDX in adult male rats reduces the tonic excitatory drive that AMPA receptors normally mediate and changes what are normally suppressive actions of NMDA receptors to ones that tonically

elevate PFC DA levels. While questions remain regarding the basis for these androgen-sensitive, estrogen-insensitive actions, one interpretation that fits existing knowledge about the physiological and anatomical links that connect key PFC glutamatergic, GABAergic and DAergic circuits--and the distributions of cognate intracellular hormone receptors within them, is that the effects on PFC DA levels reported here stem from cell-specific androgen impact on NMDA receptors. While this working hypothesis represents a decidedly reductionist view, the centrality of NMDA receptor-mediating signaling to contemporary etiological theories regarding the debilitating cognitive dysfunction in schizophrenia and other disorders e.g. autism and ADHD—where there is increased vulnerability in males (Moghaddam, 2003; Lewis and Moghaddam, 2006; Arnsten, 2009; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010) provides impetus for continuing to pursue it, as do recent findings suggesting that testosterone treatment may be effective in reducing the all too often treatment-resistant cognitive symptoms of schizophrenia in men (Strous et al., 2003; Ko et al., 2008).

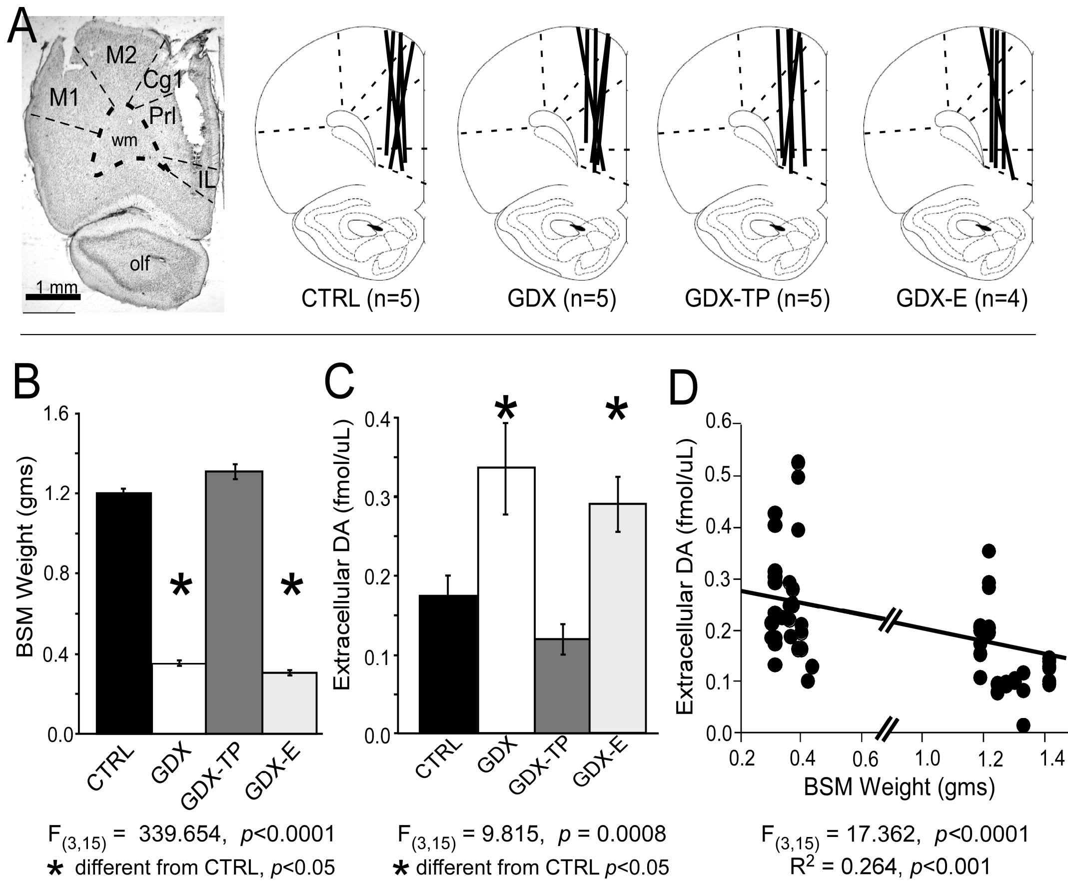

Figure IV.1. A: Representative photomicrograph showing the track of a microdialysis probe in the medial prefrontal cortex (PFC) of a control animal in the D(–)-2-Amino-5-phosphonopentanoic acid (APV) cohort. Boundaries between cytoarchitectonic fields and between cortex and white matter are marked with dashed lines. Line drawings (modified from Paxinos and Watson, 1998) illustrate the locations of microdialysis probe tracks (thick black lines) for each animal included in the sham-operated control (CTRL), gonadectomized (GDX), and gonadectomized and supplemented with testosterone propionate (GDX-TP) or 17-β-estradiol (GDX-E) APV animal groups. Numbers in parentheses below the drawings identify the number of animal subjects in that group. B., C. Bar graphs showing mean weights of the bulbospongiosus muscles (BSM, B) in grams and mean extracellular dopamine (DA) levels (fmol/μL, C) (± standard error of the mean) for CTRL (black bars) GDX (white bars), GDX-TP (dark gray bars) and GDX-E (light grey bars) groups. Mean BSM weights were significantly lower (B) and extracellular DA levels were significantly higher (C) in GDX and GDX-E rats compared to the CTRL and GDX-TP groups (asterisks). D: Regression plots that relate 20-minute bin measurements of individual animals' PFC DA levels to their BSM weight reveal that the two are significantly correlated. R^2 values appear in the upper left. Additional abbreviations: M1, motor cortex; M2, premotor cortex; Cg1, anterior cingulate cortex; Prl, Prelimbic cortex; IL, infralimbic cortex; olf, olfactory bulb; wm, white matter.

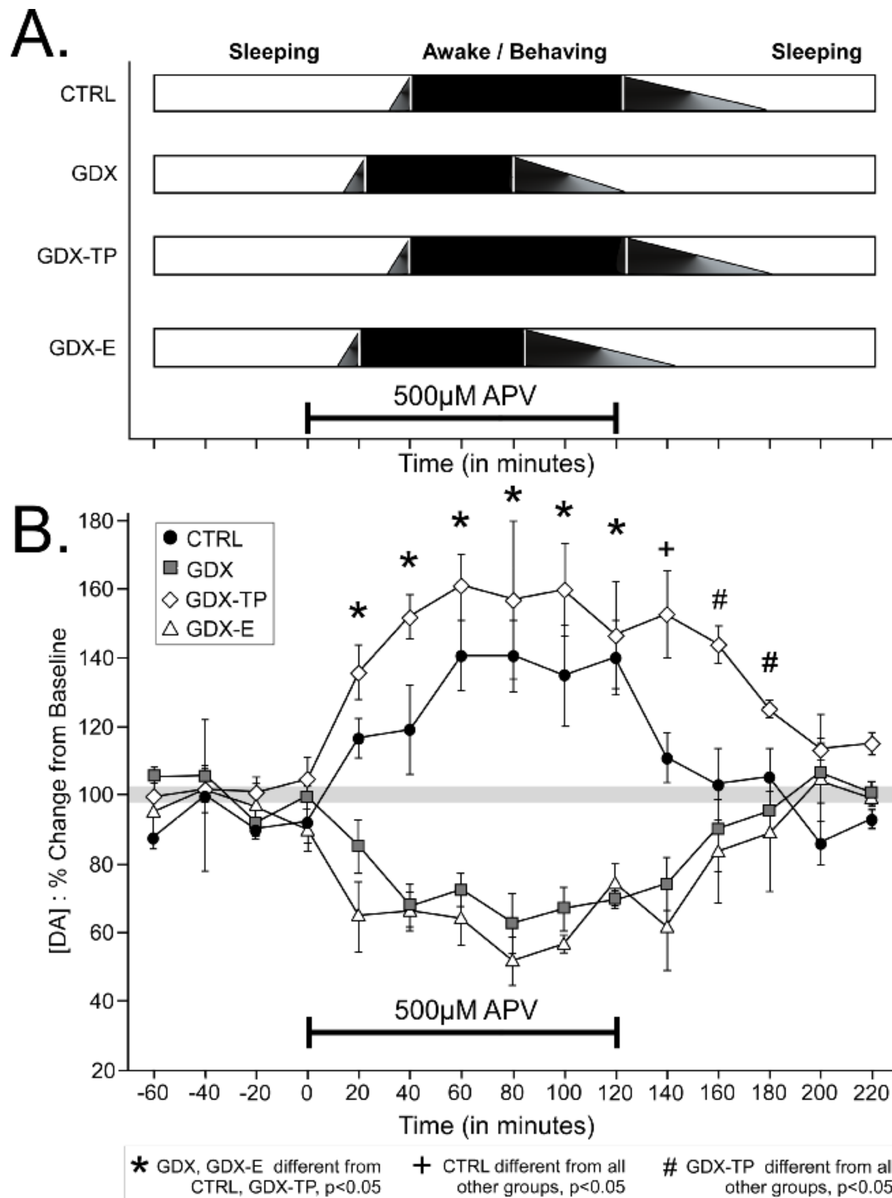

Figure IV.2. A: Timeline showing earlier onset and offset of behavioral arousal (black areas) in the gonadectomized (GDX), and gonadectomized and supplemented with 17-β-estradiol (GDX-E) animals compared to the sham-operated control (CTRL) and gonadectomized, testosterone propionate-supplemented (GDX-TP) groups induced by infusion of D(–)-2-Amino-5-phosphonopentanoic acid (APV). B: The effect of reverse-dialysis application of APV in prefrontal cortex (PFC) extracellular dopamine (DA) levels expressed as mean percent change from baseline (± standard error of the mean) in CTRL (black circles), GDX (grey squares), GDX-TP (white diamonds) and GDX-E (white triangles) groups. Asterisks indicate timepoints where drug effects on DA levels in GDX and GDX-E animals are significantly different than that in CTRL and GDX-TP groups. A plus sign (+) indicates the timepoint where DA levels in CTRL rats were significantly different from all other groups, and pound signs (#) indicate times when DA levels in GDX-TP rats were significantly different from all other groups.

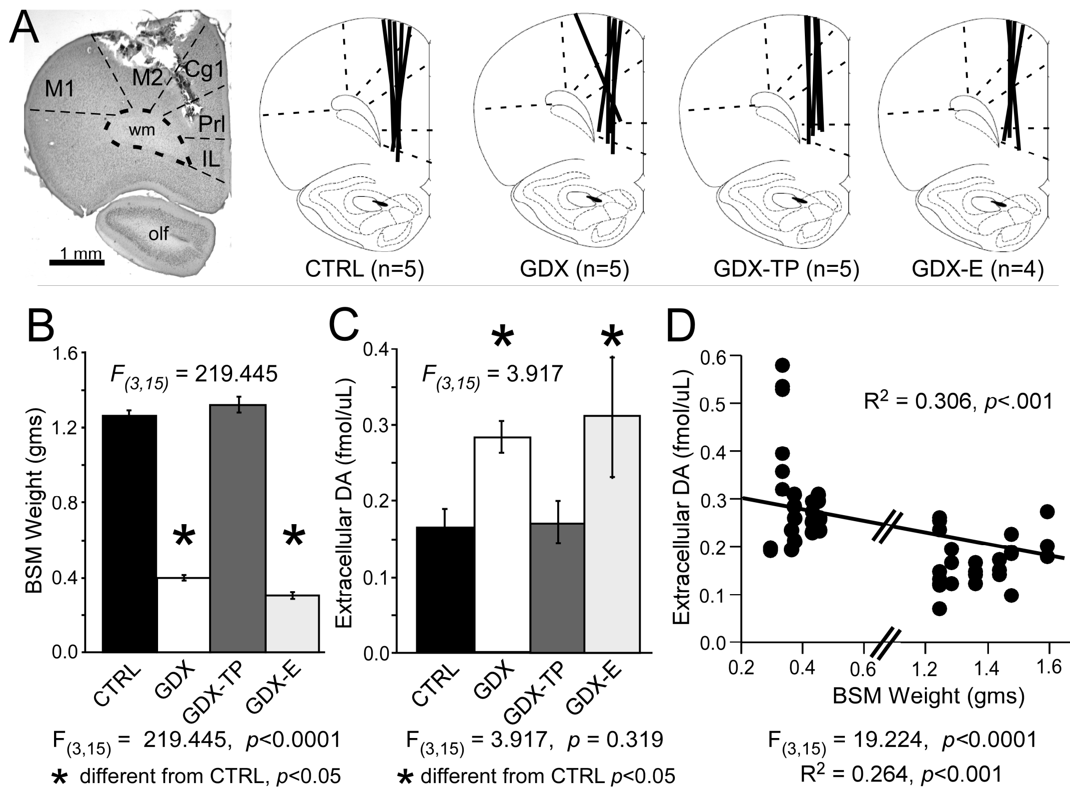

Figure IV.3. A: Representative photomicrograph showing the track of a microdialysis probe in the medial prefrontal cortex (PFC) of a control animal in the 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQCX) cohort. Boundaries between cytoarchitectonic fields and between cortex and white matter are marked with dashed lines. Line drawings (modified from Paxinos and Watson, 1998) illustrate the locations of microdialysis probe tracks (thick black lines) for each animal included in the sham-operated control (CTRL), gonadectomized (GDX), and gonadectomized and supplemented with testosterone propionate (GDX-TP) or 17-β-estradiol (GDX-E) APV animal groups. Numbers in parentheses below the drawings identify the number of animal subjects in that group. B., C. Bar graphs showing mean weights of the bulbospongiosus muscles (BSM, B) in grams and mean extracellular dopamine (DA) levels (fmol/μL, C) (± standard error of the mean) for CTRL (black bars) GDX (white bars), GDX-TP (dark gray bars) and GDX-E (light grey bars) groups. Mean BSM weights were significantly lower (B) and extracellular DA levels were significantly higher (C) in GDX and GDX-E rats compared to the CTRL and GDX-TP groups (asterisks). D: Regression plots that relate 20-minute bin measurements of individual animals' PFC DA levels to their BSM weight reveal that the two are significantly correlated. R^2 values appear in the upper left. Additional abbreviations: M1, motor cortex; M2, premotor cortex; Cg1, anterior cingulate cortex; Prl, Prelimbic cortex; IL, infralimbic cortex; olf, olfactory bulb; wm, white matter.

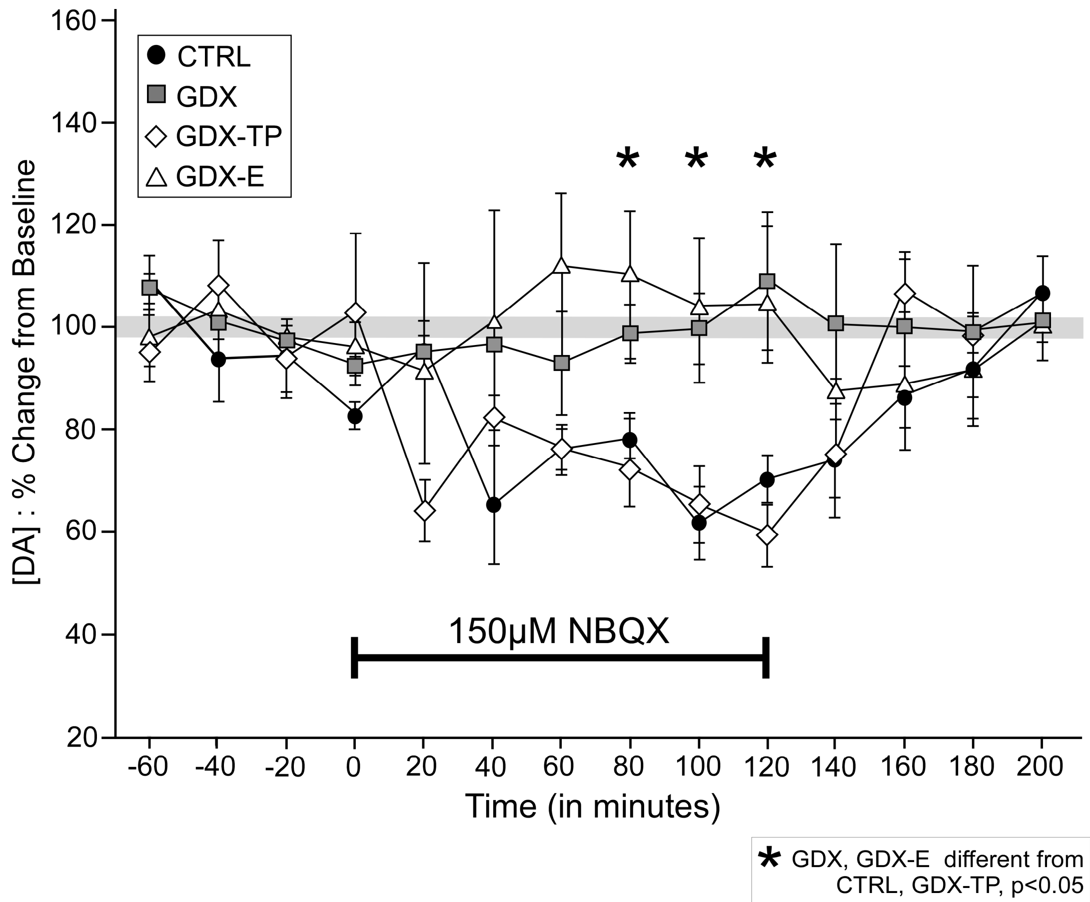

Figure IV.4. The effect of reverse-dialysis application of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) in prefrontal cortex (PFC) extracellular dopamine (DA) levels expressed as mean percent change from baseline (± standard error of the mean) in CTRL (black circles), GDX (grey squares), GDX-TP (white diamonds) and GDX-E (white triangles) groups. Asterisks indicate timepoints where drug effects on DA levels in GDX and GDX-E animals are significantly different than that in CTRL and GDX-TP groups.

General Discussion

Summary of Major Findings

The studies presented in this dissertation sought to explore the role of gonadal hormone and especially androgen modulation of the dopamine (DA) system in the prefrontal cortex (PFC) in adult male rats. Specifically, the questions asked in the initial chapters of this dissertation were: 1) Does gonadal hormone regulation of DA-dependent PFC-mediated functions extend from the orbital and medial subdivisions of the PFC (Adler et al. 1999, Kritzer et al. 2001, Kritzer et al. 2007) to the third and final division of the PFC, the perirhinal cortex? 2) Do the previously reported changes in the density of axons immunoreactivte for the DA-synthesizing enzyme tyrosine hydroxylase (TH) seen in acutely and chronically gonadectomized (GDX) adult male rats translate into the functional measure of initially decreased and then chronically increased extracellular DA levels? 3) If so, where are the androgen receptor (AR)-bearing cells that would therefore potentially influence the mesocortical DAergic pathway arising from the ventral midbrain? In answering the third question addressed in this dissertation, I found that the largest population of cells that project to the ventral tegmental area (VTA) that were also AR-immunoreactive (AR-IR) arose from glutamatergic projection neurons in the PFC itself. Thus, the final chapter addresses a fourth question raised from the course of research – 4) Are PFC glutamate/DA interactions in chronically GDX animals androgen-sensitive?

In addressing the first question, the novel object recognition (NOR) task was selected for a variety of reasons. First and foremost, this task is well-known to be sensitive to selective lesions of prefrontal areas surrounding the rhinal fissure (Aggleton et al., 1997; Barker et al., 2007; Buffalo et al., 2006; Cowell et al., 2006; Ennaceur et al., 1996, 1997; Moses et al., 2005; Mumby and Pinel, 1994; Winters et al., 2004). Secondly, since the NOR task relies on spontaneous rather than rewarded activity, previous effects seen in GDX animals on tasks requiring motivation for reward (Kritzer et. al 2007) did not factor into the hormone effects observed. The results contained in Chapter I represent the first characterization of hormone, specifically androgen, mediation of performance in the NOR task in adult male rats, thus demonstrating that gonadal steroid hormones affect DA-dependent behaviors in all three major subdivisions of the PFC in adult male rats, largely in an androgen-sensitive, estrogen-insensitive manner (Aubele et al. 2008; Kritzer et al. 2007; Kritzer et al. 2001). Given that, in all areas of the PFC, the parameter of extracellular DA level is critical for proper functioning of the local network properties (see Introduction) and that previous studies had seen an initial decrease followed by a more stable, chronic 40% increase in the density of axons immunoreactivite for TH specifically in the prefrontal but not in the adjacent somatosensory or motor cortices (Kritzer, 1999; Kritzer, 2000) in GDX adult male rats, the studies of the next chapter focused on ascertaining if this anatomical difference translated

into the more functional measure of increased PFC DA tone in these animals by using *in vivo* microdialysis in sham-operated, GDX, and GDX animals replaced with either the aromatizeable androgen testosterone propionate (GDX-TP) or estrogen (GDX-E) either 4 or 28 days post-GDX. These experiments showed that in acutely GDX animals, extracellular DA levels were initially decreased in an estrogen-reversible manner, while in chronically GDX animals, extracellular DA increased about two-fold in an androgen- but not estrogen-reversible manner, in parallel with the initially suppressive but chronically stimulatory effects of GDX on TH-immunoreactive axon density (Aubele and Kritzer, 2010). While there are many endpoints to consider that may contribute to the dysregulation in extracellular DA level seen in GDX animals, such as changes in catabolism, DA reuptake, or DA synthesis, the next chapter of this dissertation focused on the determination of possible hormone influences over the major source of PFC DA – the VTA. Since it had already been determined that the PFC-projecting DAergic cells of the ventral midbrain themselves have relatively low (4-25%) coincident labeling with immunoreactivity for AR (Kritzer and Creutz, 2008), and given the substantial effects on DA-dependent PFC behavior and extracellular DA level described in Chapters I and II of this dissertation, I explored the potential for androgen sensitivity one synapse removed from the VTA by combining neuroanatomical tract-tracing methods with androgen receptor immunocytochemistry to ask whether any of the brain areas afferent to the

VTA could also be potential sites for androgren regulation.

The results of that study found several potential brain regions that contained a significant amount of coincidence between the retrograde label cholera toxin (β subunit) and immunoreactivity for AR, but by far the highest amount of double-labeling was seen in the deep-layer pyramidal cells of the PFC itself. Thus, the final chapter in this dissertation sought to begin explore the potential for hormone sensitivity of DA/glutamate interactions in the PFC, which has never before been characterized in adult male rats. Given the complexity of receptor subtype-selective control that glutamate exerts over PFC DA, the studies in Chapter IV used reverse dialysis application of the *N*-methyl *D*-aspartate (NMDA) antagonist D (–)-2-Amino-5-phosphonopentanoic acid (APV) or the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) to analyze of the effects of chronic GDX and hormone replacement on glutamate-stimulated DA levels in the PFC. What was discovered was that, in the GDX cohort, APV produced a reversal of the normal increase in extracellular PFC DA levels seen in sham-operated animals significant decrease in extracellular PFC DA, while NBQX infusion had no appreciable effect on PFC DA levels. These findings suggested that 1) in the GDX rats there is a tonic, NMDA receptor-mediated stimulation of PFC DA level in contrast to the normally exerts suppressive effects on PFC DA tone that this receptor mediates (Moghaddam, 2003) and 2) the normally

AMPA receptor-mediated stimulation of PFC DA tone (Jedema and Moghddam, 1996; Takahata and Moghaddam, 1998) is decreased to absent in GDX animals. These effects, like those seen on basal DA levels and DA-dependent PFC tasks, were attenuated by replacement of GDX animals with androgen, but not estrogen.

The studies in this dissertation answered some questions about the nature of gonadal hormone regulation of PFC DA systems by showing that DA-dependent function in all major subdivisions of the PFC are mediated by hormones, especially androgen (Adler et al. 1999, Kritzer et al. 2001, Kritzer et al. 2007, Aubele et al. 2008). Further, previous correlations between performance in DA-dependent PFC tasks and the density of axons that are TH-IR in the PFC (Kritzer et al. 2007) were expanded to include the functional measure of extracellular levels in the PFC. Finally, the anatomical tract-tracing and *in vivo* microdialysis studies in Chapters III and IV demonstrated that the previous effects on DA-dependent PFC behaviors and PFC DA levels characterized in this dissertation and in previous studies from this lab may be secondary to hormone effects on DA/glutamate interactions in the PFC itself. Thus, in exploring the means whereby gonadal hormones could exert their critical influence over PFC function and its dysfunction in disease in adult male rats, the studies contained in this dissertation have expanded from hypotheses centered on a strict assessment of PFC DA systems (Adler et al., 1999; Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008, Aubele and Kritzer 2010) to

ones focused evaluation of glutamate/DA interactions within the PFC. However, the studies contained herein are only a small first step and many questions remain regarding the possible hormone influence over amino acid and amine neurotransmission in the PFC. The following sections will introduce some of my thoughts on some of the questions raised by the results of these studies and focus on possible future directions for continued evaluation of gonadal steroid hormone modulation of PFC DA systems.

Future Directions

The studies in this dissertation firm previous evidence for gonadal steroid hormone mediation of PFC DA systems and offer one proposed mechanism of actions whereby androgen regulation in males may ultimately affect the critical parameter of extracellular DA levels. However, if these studies have uncovered anything about the nature of PFC DA systems, it is that they are complex, with modulatory and co-modulatory interactions with other neurotransmitters as well as seemingly selective effects on certain types of PFC cells. This dissertation has focused more on the origin of PFC DA levels and endpoints that are more likely to control the rate of release of DA into the extracellular space of the PFC. While of course hormone effects on DA uptake, reuptake, and catabolism cannot be ignored, previous studies in this lab have shown that gonadal hormone regulation/GDX dysregulation of these pathways is unlikely to contribute either the basal/resting tonic elevation or to the changes in

response to glutamate-stimulated in extracellular DA levels in GDX animals observed in this dissertation (Meyers et al., 2010, Meyers and Kritzer, 2010). Rather, in view of the stimulatory effects that long-term GDX has on the density of PFC axons that are immunoreactive for the DA-synthesizing enzyme tyrosine hydroxylase (Kritzer et al. 1999; Kritzer, 2000), the information from Chapters III and IV showing that changes in DA may be driven in part by dysregulation of the presumed excitatory glutamatergic system, and given the numerous precedents set for hormone effects on DA release in subcortical centers (Castner et al., 1993; Becker and Rudick, 1999; Becker, 2000; Dluzen and McDermott, 2008), it is quite possible that perhaps more significant contributions to the dysregulation in PFC DA levels seen in GDX animals could come from dysregulation of DA synthesis and/or release. Thus, increased extracellular DA in GDX could result from increased release, increased synthesis, or both.

While there is an increase in TH-IR axon density in the PFC, what is unclear is if there is an overall increase in the absolute number of TH-IR neurons in the VTA in GDX animals as compared to control animals, which would indicate an increase in synthesis of DA in these animals. Likewise, it is also possible that there is an abnormally increased release of DA in GDX animals; this possibility would be indicated by low intracellular levels of stored DA as compared to sham-operated control animals. Thus, there is a need for testing intracellular dopamine levels as

compared to those seen extracellularly; this could be accomplished in a number of ways. For instance, a tissue-punch method could be used to estimate intracellular dopamine in the cell bodies of the VTA via HPLC detection; this method has been used in subcortical areas such as the caudate-putamen and medial preoptic area (Du et al., 1998) and in cortical regions (Gagnaire et al. 2006) in order to estimate amounts of stored dopamine in this tissue. Another method might be to use an amphetamine challenge in GDX animals while measuring PFC DA release from the medial prefrontal cortex. Previous experiments have indicated that amphetamine acts at the vesicular rather than the plasma membrane level where it redistributes dopamine to the cytosol by promoting reverse transport (through the DAT) and release of DA from storage vesicles (Sulzer et al. 1995) and that systemically administered d-amphetamine increases extracellular DA in freely-moving rats (Pum et al., 2007); the percent increase in extracellular DA stimulated by amphetamine could therefore be used to measure previously synthesized and stored DA across groups. If the percent increase in extracellular DA stimulated by amphetamine is less in GDX animals, it may indicate abnormally increased release under baseline conditions in GDX animals, and then steps could be made to identify substrates by which androgen may act to increase DA release probabilities.

Perhaps the most intriguing questions raised by the studies in this dissertation concern interactions between the DA and glutamate systems

in the PFC of adult male rats – How might a lack of androgen stimulation contribute to the dysregulation of glutamate-stimulated extracellular PFC DA levels? For instance, while the model proposed in Chapter IV of this dissertation outlines one possible way in which androgens might regulate the expression of glutamate receptor subunits specifically in the layer V projection cells of the medial prefrontal cortex, the ways in which this proposed cell-specific regulation might occur are unknown. While DA, especially at D_1 receptors, has been shown to increase the cell surface expression of NMDA and AMPA receptors acutely in culture (Gao and Wolf, 2008; Sun et al. 2008; Sun et al. 2005), far less is known about the interaction of these D_1 and glutamate receptor subtypes over chronic timelines or exactly how androgens might mediate AMPA or NMDA receptor expression in the PFC. Further, glutamate does not only stimulate receptors in these deep-layer pyramidal cells; in fact, NMDA receptors in particular are much more abundant on the fast-spiking, parvalbumin (PV)-positive interneurons in PFC (Pratt et al., 2008, Lisman et al. 2008) than on the pyramidal cells. While these fast-spiking GABAergic neurons do not contain AR but rather intracellular estrogen receptor (Kritzer, 2006), the contribution of these local circuit interneurons to mediating the glutamatergic drive of the PFC to the VTA cannot be overlooked – especially since some DA-dependent PFC functions have been found to be estrogen-mediated in adult male rats (Kritzer et al. 2007). Further, interactions between DA D_1 receptor subtypes and both

AMPA and NMDA receptors at any or all of these cell types could also be evaluated in sham-operated and GDX animals in order to determine if the well-characterized interactions between these receptors are altered in these animals. For instance, the well-described NMDA-D_1 receptor “trap,” in which activation of one receptor affects the function of the other through close physical interactions (Scott et al. 2006, Cepeda and Levine, 2006), demonstrates that alterations in especially NMDA receptor function might change the efficiency of DA stimulation via D_1-type receptors, which are by far the most abundant in the PFC (Santana et al. 2009). One way that this might occur is through altered subunit composition of NMDA receptors. For instance, in CA1 pyramidal neurons, D_1-type receptor activation elicits a long-term plasticity of NMDA receptor-mediated synaptic currents with the polarity of plasticity determined by NMDA receptor NR2A/B subunit composition *in vitro*, such that synaptic responses mediated by NMDA receptors that include NR2B subunits are potentiated by D_1-type receptor activation receptor activation and responses mediated by NMDA receptors that include NR2A subunits are depressed (Stramiello and Wagner, 2008; Valera et al. 2009). Thus, an alteration in either the number of NMDA receptor subunits available or their subunit composition can change the amount and/or actions of DA at D_1-type receptors. Accordingly, there is a need for future evaluation of the effects of GDX on AMPA and especially NMDA receptor surface expression and subunit composition in the different cell types of the PFC that are involved in the regulation of

extracellular DA level– that is, in pyramidal cells, PV-IR GABAergic interneurons and other types of GABAergic cells. Some of this work has very recently been initiated in our lab – immunohistochemistry for the NR-1 subunit of the NMDA receptor is currently being compared in PV-IR and non-PV labeled cells in the PFC of sham-operated, GDX, GDX-TP and GDX-E animals; while the data are still very preliminary, these anatomical studies will aid in evaluation of possible mechanisms of hormone regulation of PFC glutamatergic systems and possibly identify more specific sites of actions where hormones may play a role.

However, while it is important to consider and understand the molecular, cellular and physiological bases of hormone actions in the PFC, it is also important to take a step back and consider how these mechanisms might be relevant to the neurological function of this system and its dysfunction in human psychiatric diseases such as schizophrenia.

Clinical Implications of Identified Effects on DA-Dependent PFC Function and Physiology

The major findings of this dissertation were that GDX impairs DA-dependent function, increases baseline extracellular DA levels, and disrupts glutamate-stimulated levels of extracellular DA in the PFC, all in an androgen-sensitive, estrogen-insensitive manner in adult male rats. An original interest in the neurobiological basis for hormone impact on the higher-order functions of the PFC and their disproportionate and disproportionately severe dysfunction in males in schizophrenia and other

disorders had led this lab and myself to focus narrowly on DA as an endpoint of gonadal hormone stimulation in male animal models. However, these experiments lead to a broadening of interest from strictly dopamine-centered hypotheses into ones that encompass DA and glutamate in hormonal control over PFC systems. The modulatory and co-modulatory interactions between these two neurotransmitters in regulating the tonic and burst-firing modes of the VTA (see Introduction) are key in maintaining basal PFC DA levels and for phasically augmenting prefrontal DA levels, respectively, in hormonally intact animals. These reciprocal activity patterns are critical and, in part, monosynaptically regulated by glutamate (see Grace, 1987; Overton and Clark, 1992) released from afferents including those coming from the PFC itself (Carr and Sesack, 2000). The interdependent, reciprocal nature of these DA/glutamate interactions in the operations of the mesocortical DA system has been demonstrated in a variety of functional and behavioral assays in rats (Verma and Moghaddam, 1996, Moghaddam et al. 1997, Takahata and Moghaddam, 1998). Perhaps not surprisingly, and in parallel with the expansion of the hypotheses in this dissertation from DA-centered to DA/glutamate interactive, anomalies in DA/glutamate interactions are also at the heart of more current etiological theories of schizophrenia, as it has been posited that the negative, PFC symptoms of this disorder may be explained by primary defects in NMDA receptor-mediated glutamate hypofunctioning, with secondary effects on PFC DA tone (Moghaddam,

2003; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010). Taken together, these findings indicate that glutamate inputs arising from PFC and other regions are critical to the mesocortical DA system, and serve as prime controllers of midbrain DA cell activity and the related functional measures of PFC DA levels. Thus, the purported hormone actions at glutamatergic endpoints proposed in this dissertation are both neurobiologically and clinically appealing. While many questions remain regarding the basis for these androgen-sensitive effects, one interpretation that fits both existing and new knowledge gleaned from studies in this dissertation about the physiological and anatomical links that connect key PFC glutamatergic and DAergic circuits is that the effects on PFC DA levels reported here could stem from cell-specific androgen impact on glutamate receptors of the NMDA subtype in PFC pyramidal neurons. Thus, the centrality of NMDA receptor-mediating signaling to the debilitating cognitive dysfunction in schizophrenia and other disorders such as autism and ADHD—where there is increased vulnerability in males (Moghaddam, 2003; Lewis and Moghaddam, 2006; Arnsten, 2009; Scott and Aperia, 2009; Seeman, 2009; Marek et al., 2010) – and recent findings suggesting that testosterone treatment may be effective in reducing the cognitive symptoms of schizophrenia in men (Strous et al., 2003; Ko et al., 2008) add to the spirit of this dissertation as well, and provide impetus for continuing to expand knowledge about gonadal hormone regulation of PFC DA systems

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