

**Sex differences in impulsivity in prepubertal and adult rats**

AN ABSTRACT

SUBMITTED ON THE FIFTEENTH DAY OF APRIL 2014

TO THE DEPARTMENT OF PSYCHOLOGY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

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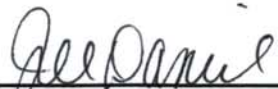
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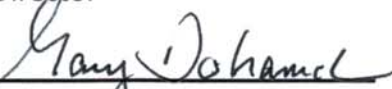
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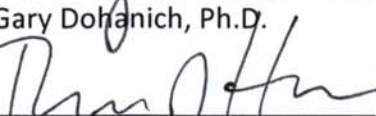
  
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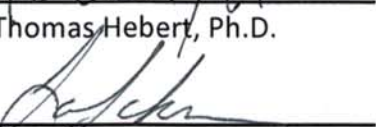
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## **Abstract**

The current set of experiments was designed to test the hypothesis that there is a sex difference in impulsivity and in brain areas associated with impulse control in prepubertal and adult rats, such that females have greater inhibitory control than do males. Preliminary studies established that neonatal testosterone exposure is able to masculinize and increase impulsive behavior in prepubertal female rats. In the current study, male and female prepubertal rats exposed to treatments that resulted in either neonatal androgen or estrogen receptor activation made more impulsive choices than did control females and their performance mirrored that of control males. Assessment of impulsivity in adult rats indicated that impulsive choice behavior was similar in males and females whereas impulsive action behavior was greater in males than in females. Analysis of protein levels of markers of dopaminergic and noradrenergic reuptake in the orbital frontal cortex (OFC) and dorsal striatum (dSTR), two brain areas important for impulse control, revealed no differences between male and female prepubertal or adult rats, whereas analysis of protein levels of markers of myelination in the OFC and dSTR revealed similar levels between the sexes in prepubertal rats but increased myelin levels in the OFC but not dSTR of adult female rats as compared to males. Furthermore, analysis of the projections from the OFC to dSTR discovered that the strength of these projections was significantly greater in adult females as compared to males. However, inactivation of the OFC during an impulsive action task in adult rats failed to have an effect on impulsive action responding. Collectively, these results demonstrate for the first time that there is a sex difference in impulsive choice control in prepubertal rats

that is organized neonatally by actions of both androgens and estrogens, this sex difference subsides in adulthood, but a sex difference in impulsive action control is present in adulthood. Furthermore, the novel discovery that adult female rats have increased levels of myelination within the OFC and increased strength of projections from the OFC to dSTR as compared to males establishes a molecular sex difference that could underlie the enhanced impulse control in females.

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A DISSERTATION

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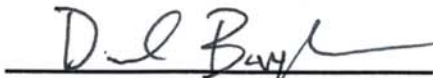
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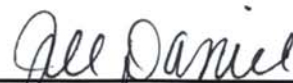
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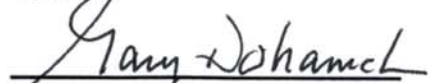
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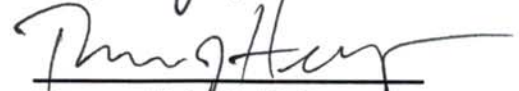
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## **Introduction**

The ability to inhibit a behavior or action is essential for performing even the simplest everyday task. Impulsive actions and decisions often lead to undesirable outcomes, and impulsivity problems contribute to many psychological disorders, such as drug addiction (Moeller et al. 2001), pathological gambling (Steel and Blaszczynski 1998), and attention deficit-hyperactivity disorder (ADHD) (Breedlove et al. 2007). Impulsivity can be defined as action without forethought and can be classified into at least two distinct processes: impulsive action and impulsive choice (Evenden 1999). Impulsive actions arise from a lack of behavioral inhibition resulting in an inability to control or suppress premature or inappropriate actions (Eagle and Baunez 2010). Impulsive action is commonly measured in humans and rodents using stop-signal tasks, during which subjects must inhibit a previously rewarded response, and using serial reaction time tasks, during which subjects must suppress an inappropriate response until the proper time (Eagle and Baunez 2010; Harrison et al. 1999). On the other hand, impulsive choices stem from making decisions or choices without appropriate deliberation of the alternative options (Eagle and Baunez 2010). Impulsive choice is often demonstrated as an aversion to a delayed reward (Dalley et al. 2008). It is commonly measured in humans and rodents using delay-discounting paradigms where impulsive choice is defined as the selection of a small immediate reward over a larger but delayed reward (Cardinal et al. 2004).

Numerous studies investigating the neuroanatomical circuitry of impulsivity have demonstrated the important role that the prefrontal cortex (PFC) plays in inhibitory

control in humans and animals (Brass and von Cramon 2002; Cardinal 2006; Dove et al. 2000). Human subjects with damage to the PFC display impulsive action during stop-signal tasks (Aron et al. 2003) and impulsive choice during gambling tasks (Bechara et al. 1994). It has been proposed that the PFC influences impulsivity by modulating the operations of lower brain areas involved in reward-based behaviors, such as the striatum (Galvan et al. 2006; Peper et al. 2012; Perry et al. 2011). The striatum is involved in reward associated stimulus-response behaviors (Eichenbaum 2012). In this view, the PFC acts as a top-down modulator of these lower brain areas, integrating behaviorally relevant information and preventing the over-reliance on fixed action patterns (Perry et al. 2011; Peters and Buchel 2011). Using functional magnetic resonance imaging (fMRI), the striatum has been shown to be hyper-responsive when individuals choose immediate rewards over delayed rewards (McClure et al. 2004), and the magnitude of striatal activation correlates with the amount of impulsive choices individuals make during a delay-discounting task (Hariri et al. 2006). In addition, using transcranial magnetic stimulation (TMS) to disrupt the PFC leads to an increase in preference for immediate small rewards over delayed large rewards (Figner et al. 2010), suggesting that blocking the ability of the PFC to modulate the operations of lower brain areas results in increased impulsivity. Furthermore, results using tract-based diffusion tensor imaging have revealed that lower integrity within the frontostriatal white matter tract predicts a greater increase in impulsivity as the delay for a large reward over an immediate small reward increases (Peper et al. 2012).

## **Prefrontal Cortex and Striatum**

Imaging studies in humans and systematic lesion studies in rats have begun to dissociate different regions of the brain underlying the different aspects of impulsivity (Eagle and Baunez 2010). In the PFC and striatum, lesion studies provide evidence that the neurobiology underlying impulsive action and impulsive choice may be dissociable (Eagle and Baunez 2010; Winstanley et al. 2006). In order to discuss the role of the PFC and striatum in impulsivity, a brief overview of the anatomy and neurochemistry of the PFC and striatum will be given.

### *Prefrontal cortex anatomy*

The extent to which substructures of the PFC are similar across species is controversial. The PFC shows species-specific variation in relative size, anatomical cytoarchitecture, neurochemistry, and connectivity (Uylings et al. 2003). Multiple nomenclatures and subdivisions of PFC structures have been proposed and used. The most common definitions will be used here to describe PFC areas in both primates and rodents (Perry et al. 2011; Price 2007). Many cross-species differences exist. However, there are also many cross-species similarities, which allow researchers to compare and generalize behavioral results across rodents and primates.

The primate and rodent PFC receives inputs from all sensory systems and most of the cerebral cortex as well as all of the major subcortical systems, such as the striatum, amygdala, cerebellum, and hippocampus (Dalley et al. 2004; Groenewegen et al. 1997). The PFC is not directly connected with primary sensory areas, but is

interconnected with secondary and association sensory cortices. Most inputs from the major subcortical systems arrive to the PFC via the thalamus. The PFC in turn projects to the striatum, amygdala, cerebellum, hippocampus, and other regions of the cerebral cortex (Dalley et al. 2004; Groenewegen et al. 1997). The primate PFC cortex is commonly divided into three cytoarchitecturally distinct networks: the medial, lateral, and orbital (Ridderinkhof et al. 2004). All three networks have been implicated in impulsivity (Crews and Boettiger 2009). The medial region is commonly further divided into the medial prefrontal cortex (mPFC) and anterior cingulate cortex (ACC). These medial regions, along with the orbital frontal cortex (OFC), likely represent similar structures in primates and rodents. (Perry et al. 2011). The rodent cortex is exclusively agranular, as compared to primate cortex that is made up of agranular, granular, and dysgranular regions (Barbas and Pandya 1989). Therefore, equivalent regions in the primate and rodent PFC are determined by connections with other brain areas and interconnections within the PFC (Perry et al. 2011). The primate lateral prefrontal cortex (LPFC) does not generalize directly to the rodent based on connectivity (Price 2007). Many researchers have suggested that the LPFC may be unique to human and non-human primates (Brown and Bowman 2002). Therefore, the term is used for primates and not for rodents, and the rodent PFC is commonly divided into just the medial and orbital networks (Price 2007). These networks have similar connectivity with those of the primate PFC, allowing researchers to compare and generalize behavioral results across species.

The primate and rodent mPFC and ACC have numerous intra-cortical connections (Perry et al. 2011). The rodent mPFC refers to various structures located along the medial wall of PFC (Dalley et al. 2004). The precentral cortex (PrC) and ACC together are often referred to as the dorsal mPFC, whereas the prelimbic (PrL), infralimbic (IL), medial orbital (MO), and ventral orbital (VO) cortices together are referred to as the ventral mPFC (Price 2007). The primate and rodent mPFC have direct projections to other brain regions that have been implicated in impulsivity. For example, the mPFC sends projections to the dorsal striatum, nucleus accumbens, and ventral tegmental area (VTA) (Vertes 2006). The mPFC also sends projections to the ACC and OFC, allowing for communication between the different PFC regions (Vertes 2006). The mPFC and ACC also receive inputs from the hippocampus, amygdala, and other limbic areas via the thalamus (Price 2007; Vertes 2006). The ACC is a target region for dopamine projections originating from the midbrain VTA forming part of the mesocortical dopamine pathway, which is strongly implicated in reward seeking and impulsivity (Meyer and Quenzer 2005). Lesions of the mPFC and ACC generally increase impulsive action but have less of an effect on impulsive choice in male rats (Eagle and Baunez 2010; Winstanley et al. 2006).

The primate and rodent OFC receives inputs from numerous cortical and subcortical areas and is highly interconnected with other PFC regions (Perry et al. 2011). Functional subdivisions within the primate OFC are divided according to the sensory modality providing input to that region. The primate OFC receives visual, somatosensory, olfactory, gustatory and visceral information from secondary sensory

cortices (Perry et al. 2011). The rodent OFC is part of the olfactory system but also receives visual and somatosensory information (Price 2007). The rodent OFC is comprised of the ventral lateral orbital (VLO), lateral orbital (LO), and ventral agranular insular (AIV) cortices (Price 2007). The VLO and LO project directly to and receive indirect projections from the dorsal striatum and nucleus accumbens via the thalamus (Schilman et al. 2008). Similar to the ACC, there are reciprocal inputs with the hippocampus and amygdala (Kita and Kitai 1990). The primate and rodent OFC also receives projections from midbrain nuclei associated with different neurotransmitter systems involved in impulsivity, such as dopamine from the VTA and norepinephrine from the locus coeruleus (Groenewegen 1988; Perry et al. 2011; Ray and Price 1992). Lesions to the OFC generally increase impulsive action but have mixed effects in terms of the direction of effect on impulsive choice with some studies reporting an increase and others reporting a decrease in impulsive choice responding in male rats (Eagle and Baunez 2010; Winstanley et al. 2004). Collectively, these studies demonstrate that the OFC plays an important role in both aspects of impulsivity and that removal of OFC output results in an alteration in impulsive choice responding.

### *Striatum anatomy*

The striatum is part of the basal ganglia and is comprised of the caudate, putamen, nucleus accumbens, and olfactory tubercle (Ragozzino 2007). Numerous fiber tracts pass through these structures giving the area a striped appearance, which inspired the name striatum (Ragozzino 2007). The primate and rodent striatum receives

input from all major areas of cortex as well as major subcortical systems, such as the hippocampus and amygdala (McGeorge and Faull 1989). The striatum is commonly divided into the dorsal and ventral striatum. The dorsal striatum (dSTR) is composed of the caudate and putamen, and the ventral striatum is composed of the nucleus accumbens and olfactory tubercle (Ragozzino 2007). The dSTR is involved in reward associated stimulus-response behavior (Eichenbaum 2012), and the ventral striatum is involved in reward-seeking behavior (Galvan et al. 2006). Lesions of the ventral striatum increase both impulsive choice and impulsive action (Cardinal et al. 2001; Christakou et al. 2004).

The rodent dSTR is not as clearly delineated into the caudate and putamen as is the primate dSTR due to the internal capsule not being as compacted and organized in rodents as it is in primates (Ragozzino 2007). Although there is no distinct border between the dorsomedial and dorsolateral striatum in the rodent, the dorsolateral and dorsomedial areas of the striatum have been implicated in different aspects of stimulus-response learning. The dorsolateral striatum (dlSTR) is critical for learning and expressing stimulus-response associations and egocentric-response information (Ragozzino 2007). The dorsomedial striatum (dmSTR) facilitates learning when conditions require inhibition of a previously relevant strategy and the learning of a new strategy. The dmSTR is critical for flexible shifting of response patterns and the maintenance of a new correct strategy once generated (Ragozzino 2007). Lesions of the dmSTR increase impulsive action during the 5-choice serial reaction time task (5-CSRTT),

and lesions of the dorsolateral striatum cause rats to be unable to perform the task (Rogers et al. 2001).

### *Neurochemistry of the prefrontal cortex*

Dopamine and norepinephrine are strong modulators of PFC activity and are both implicated in impulse control (Dalley et al. 2008). Therefore, these two neurotransmitter systems are one focus of the current research. Dopamine staining is present in most areas of the rodent PFC (Van De Werd et al. 2010). Dopamine input arrives into the PFC from the VTA through the mesocortical pathway (Meyer and Quenzer 2005). There are five subtypes of dopamine receptors: D<sub>1</sub>-D<sub>5</sub>. D<sub>1</sub> and D<sub>5</sub> are excitatory metabotropic receptors, and D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub> are inhibitory metabotropic receptors (Meyer and Quenzer 2005). Dopamine is removed from the synapse by the dopamine transporter (DAT) and metabolized by MAO (Meyer and Quenzer 2005). Dopamine utilization is increased in the OFC during performance on a delay-discounting task (Winstanley et al. 2006).

In humans, psychostimulants such as amphetamine or methylphenidate, which prevent dopamine reuptake and increase dopamine release from dopaminergic terminals, are highly effective in decreasing impulsivity among individuals with ADHD (Arnsten 2009). However, laboratory studies in male rodents find mixed results reporting that dopamine agonists can either decrease (Richards et al. 1999; Sagvolden et al. 1992) or increase impulsive choice (Charrier and Thiebot 1996; Evenden and Ryan 1996). The discrepant results of these studies are believed to be due to various factors,

such as the dose used, baseline level of impulsive choice behavior, and different methodological details of the task (Perry and Carroll 2008). The role of dopamine in the PFC in impulsive action remains unclear, with systemic administration of nonspecific dopamine agonists yielding inconsistent results on impulsive action (Perry and Carroll 2008). In humans, psychostimulants tend to decrease impulsive action (Perry et al. 2011). These effects are most likely moderated by  $D_1$  and  $D_2$  receptors, as antagonism of either subtype increases impulsive action (Passeti et al. 2003). Studies report mixed findings in terms of the direction of the effects of dopamine in the PFC on impulsivity. However, most studies demonstrate that dopamine levels in the PFC strongly modulate impulsive choice and impulsive action.

Central norepinephrine neurons in the locus coeruleus project diffusely to almost every part of the brain, including the PFC (Perry et al. 2011). Norepinephrine cell bodies receive reciprocal innervation from the mPFC (Heidbreder and Groenewegen 2003). Norepinephrine release into the synapse is deactivated by rapid uptake by the norepinephrine transporter (NET) into the presynaptic terminal, followed by monoamine oxidase (MAO) metabolism (Meyer and Quenzer 2005). Four families of receptors are activated by norepinephrine:  $\alpha_1$ -adrenergic,  $\alpha_2$ -adrenergic,  $\beta_1$ -adrenergic, and  $\beta_2$ -adrenergic receptors (Bylund et al. 1994).  $\alpha_1$  are phosphoinositide systems receptors,  $\alpha_2$  are inhibitory metabotropic receptors, and  $\beta_1$  and  $\beta_2$  are excitatory metabotropic receptors (Meyer and Quenzer 2005). Most research has focused on the  $\alpha_2$ -adrenergic receptors because ADHD is proposed to involve a deficit in PFC  $\alpha_2$ -adrenergic activity (Arnsten 2000).

Norepinephrine has been shown to play a role in both impulsive choice and impulsive action. Activation of  $\alpha_2$ -adrenergic receptors by agonists decreases impulsive choice and impulsive action in male mice (Franowicz et al. 2002), rats (Tanila et al. 1996), monkeys (Rama et al. 1996), and humans (Jakala et al. 1999). Impulsive choice is also decreased by elevating extracellular norepinephrine levels via NET inhibition (Blondeau and Dellu-Hagedorn 2007; Robinson et al. 2008). In addition, NET inhibition decreases impulsive action on the 5-CSRTT and stop-signal tasks in rodents (Robinson et al. 2008). Similar findings are seen in healthy humans. The NET inhibitor atomoxetine decreased impulsive action during a stop-signal task in humans (Chamberlain et al. 2006). Antagonism of  $\alpha_2$ -adrenergic receptors increases impulsive action during the 5-CSRTT task and increases phosphorylation of CREB in the OFC (Koskinen et al. 2003; Sun et al. 2010). These results suggest that  $\alpha_2$ -adrenergic receptors, perhaps in the OFC, play an important role in both types of impulse control. With some exceptions, the majority of studies indicate that increased norepinephrine levels in the PFC lead to decreased impulsive choice and impulsive action.

### *Neurochemistry of the striatum*

The striatum is comprised predominantly of medium-size spiny neurons, which represent the principle output neurons of the striatum and contain the neurotransmitter GABA (Smith and Bolam 1990). The main source of dopamine in the striatum originates from the substantia nigra pars compacta, forming the nigrostriatal pathway (Meyer and Quenzer 2005). Dopamine actions in the striatum can directly

affect medium spiny neurons and corticostriatal input (Bamford et al. 2004; Voulalas et al. 2005). All types of dopamine receptors,  $D_1$ -  $D_5$ , have been found in the striatum (Nicola et al. 2000). Dopamine depletion in either the dorsal or ventral striatum has been shown to have no effect on impulsive action in male rats during the 5-CSRTT (Baunez and Robbins 1999; Cole and Robbins 1989). However, the lack of an effect of dopamine depletion may result from the opposing roles of excitatory  $D_1$  and inhibitory  $D_2$  receptors in the striatum. Blocking  $D_1$  receptors, thereby reducing activity, in the dmSTR decreases impulsive action, whereas blocking  $D_2$  receptors, thereby increasing activity, in the dmSTR increases impulsive action in male rats during the stop-signal task (Eagle and Baunez 2010). These results support the idea that increased dSTR activity leads to increased impulsive action.

Projections from the striatum diverge into two routes: the direct and indirect pathways (Graybiel 2000; Mink 1996). The direct pathway leads from the striatum into the globus pallidus internal (GPi) and the substantia nigra pars reticulata (SNpr). These regions then send inhibitory projections to the thalamus. The thalamus then projects to the motor cortex which leads to behavioral output. Because the GABAergic output of the striatum is inhibitory, the inhibition of neurons in the GPi and SNpr result in a disinhibition of the thalamus, making the direct pathway effectively excitatory (Miller and Buschman 2007). The indirect pathway leads from the striatum into the globus pallidus external (GPe), which in turn projects to the subthalamic nucleus (STN), which projects onto the GPi and SNpr. GPe inputs into STN are inhibitory. However, STN inputs into the GPi and SNpr are excitatory (Miller and Buschman 2007). Because there is one

added inhibitory synapse, the indirect pathway results in an overall inhibition of the thalamus and its projections to motor cortices. These two pathways allow for the release of desired behavioral patterns while inhibiting undesired behavioral patterns (Miller and Buschman 2007).

### *Prefrontal cortex and striatum connectivity*

Corticostriatal projections from the OFC to the dSTR are strongly implicated in impulse control (For review, see Eagle and Baunez 2010). Therefore, the OFC and dSTR are the two main brain areas of focus in the current studies. As previously discussed, it has been proposed that inhibitory control results from the PFC modulating the operations of lower brain areas involved in reward-based behaviors, such as the striatum (Galvan et al. 2006; Peper et al. 2012; Perry et al. 2011). Supporting this idea is the finding that disruption of PFC output by transcranial magnetic stimulation (TMS) leads to increased impulsivity (Figner et al. 2010). The excitatory glutamatergic inputs of the OFC into the dSTR are hypothesized to activate the indirect pathway resulting in the suppression of an undesired behavior (Eagle and Baunez 2010). As mentioned previously, lower integrity within the frontostriatal white matter tract is associated with a greater increase in impulsivity as the delay for a large reward over an immediate small reward increases (Peper et al. 2012). In the central nervous system (CNS), white matter or myelin is formed by oligodendrocytes that wrap tightly around axons insulating and increasing the speed of neurotransmission (Sherman and Brophy 2005). The ratio of the axon diameter to myelin diameter (g-ratio) is fairly consistent across myelinated

neurons, meaning that larger axons have thicker myelin, and vice versa (Sherman and Brophy 2005). Therefore, increased levels of white matter could be indicative of either a greater number of neuronal connections or a greater average axonal diameter of neurons. Both the number of neuronal connections and axonal diameter are associated with faster, more efficient neuronal communication. Increased strength of projections from the OFC to the dSTR or increased myelination of the frontostriatal tract may lead to increased control of the OFC over the dSTR leading to increased inhibition (via the indirect pathway) of thalamic projections to motor cortex thereby dampening motor output and enhancing the ability to inhibit undesirable behaviors.

### **Sex Differences in the Brain and Behavior**

Sex differences have been documented in anatomy, neurochemistry, and physiology throughout the brain (For review, see Andreano and Cahill 2009). For example, when controlling for relative brain size, the hippocampus is larger in women than in men (Filipek et al. 1994), and the amygdala is larger in men than in women (Giedd et al. 1996). Sex differences have also been reported in dopamine levels (Lavalaye et al. 2000; Mozley et al. 2001), serotonin levels (Ortiz et al. 1988), and GABA levels (Sanacora et al. 1999). Studies have shown that the overall ratio of gray matter to white matter is slightly higher in women than in men (Allen et al. 2003; Goldstein et al. 2001; Gur et al. 1999). However, the amount of gray and white matter can vary between the sexes by brain area (Goldstein et al. 2001). For example, an MRI study examining gray and white matter in frontal and parietal brain areas that were correlated with

intelligence quotient (IQ) scores revealed that men had 6.5 times the amount of gray matter as did women and women had 9 times the amount of white matter as did men in these brain areas related to intellectual functioning (Haier et al. 2005).

In addition to changes in the brain, biological sex and sex hormones have been shown to influence many cognitive behaviors (For review, see Luine and Dohanich 2008). For example, women outperform men on tasks of verbal memory (Kramer et al. 1997; Mann et al. 1990; Portin et al. 1995) and recall of episodic events (Bloise and Johnson 2007). Men solve mental rotation and navigation tasks faster and more accurately than do women (Astur et al. 2004; Levine et al. 1999; Postma et al. 2004; Silverman, I et al. 2000). Moreover, different neural networks are activated during mental rotation tasks in men and women. Men show increased activity in the parietal lobe, and women show increased activity in the frontal lobe (Thomsen et al. 2000; Weiss et al. 2003). This indicates the possible use of different strategies employed by men and woman to solve the task which most likely leads to the sex difference seen in the behavior. In rodents, males outperform females on spatial memory tasks, such as the radial arm maze and Morris water maze (Gresack and Frick 2003; Luine and Rodriguez 1994; Williams et al. 1990). However, these sex differences can vary with age, stress, and task demands (Bucci et al. 1995; Markowska 1999; Perrot-Sinal et al. 1996), indicating that differences in strategies used to solve the tasks are again most likely contributing to the sex differences in the behavior. Sex differences in strategy selection must result from a sex difference in brain anatomy, chemistry, or connectivity.

The many findings like those discussed above indicate a strong role for biological sex in brain development and behavior. Most of these animal studies have focused on learning and memory with little focus on sex differences in PFC-dependent behavior. As previously discussed, the PFC is an important brain area for many cognitive and executive functions, including inhibitory control (Dalley et al. 2004; Eagle and Baunez 2010). Although less research has been conducted on sex differences in impulsivity, some studies along with sex differences in the incidence rates, age of onset, course, and symptomatology of some impulsivity disorders indicate that sex differences in impulsivity may exist. Sex differences in impulsivity disorders will be discussed first, followed by differences seen in the general population.

#### *Sex differences in human impulsivity disorders*

Attention deficit-hyperactivity disorder (ADHD) is a common neurodevelopmental disorder (Polanczyk et al. 2007) with a strong genetic basis (Wallis et al. 2008), characterized by attention deficits, pathological impulsivity, and extreme hyperactivity. Individuals with ADHD are typically diagnosed as having one of three subtypes: the inattentive subtype, the hyperactive-impulsive subtype, or the combined subtype (Polanczyk et al. 2007). The prevalence rate of ADHD is higher for males than it is for females (Holden 2005; Swanson et al. 1998). Furthermore, the inattentive subtype is most common in girls (Biederman et al. 2002), whereas the hyperactive-impulsive and combined subtypes are most common in boys (Adler et al. 2008).

Studies examining performance during impulsivity tasks in male and female ADHD patients have yielded mixed results. One study found no significant sex difference in impulsive action during a Stroop task and a stop signal task (Rucklidge and Tannock 2002). However, a meta-analysis of stop-signal studies found a small effect of sex, whereby impulsivity in male ADHD patients as compared to male controls was more severely impaired than it was in female ADHD patients as compared to female controls (Lipszyc and Schachar 2010). Common treatments for ADHD, such as amphetamine and methylphenidate, appear to be equally effective in both sexes (Rucklidge 2010). However, a recent study reported better outcomes in females as compared to males with a newer ADHD treatment, atomoxetine, which acts primarily as a norepinephrine reuptake inhibitor (Marchant et al. 2011).

Addictive behaviors, such as gambling and drug abuse, can be conceptualized as impulsive choices, whereby smaller immediate rewards are favored over larger delayed rewards (Petry and Casarella 1999). Pathological gambling, the maladaptive behavior of gambling in spite of adverse consequences (Alessi and Petry 2003), is characterized by high levels of impulsivity (Steel and Blaszczynski 1998) and frequently co-occurs with ADHD (Derevensky et al. 2007). Pathological gambling is more common in men than in women, and the age of onset is earlier in men than in women. However, women have a quicker progression to pathological gambling than do men (Johansson et al. 2009; Tavares et al. 2001).

Similar to pathological gambling, drug abuse is closely associated with impulsive behavior (Perry and Carroll 2008), such that drug abusers are significantly more

impulsive than controls (Kirby et al. 1999; Madden et al. 1997). Drug abuse frequently co-occurs with ADHD (Schubiner 2005). Sex differences are reported in many aspects of human and animal drug abuse (Becker and Hu 2008; Roth et al. 2004). For example, the rate of escalation of drug use and risk of relapse following abstinence is greater in females compared to males (Brady and Randall 1999; Carpenter et al. 2006; Mann et al. 2005). In addition, in both humans (Lejuez et al. 2007) and rodent models (Anker et al. 2008) greater impulsivity is found amongst drug abusing females as compared to drug abusing males.

These sex differences in prevalence rates and course of impulsivity disorders indicate that sex differences in impulsivity may exist. The higher prevalence rates of ADHD and pathological gambling in males as compared to females suggest that males may be more susceptible than females to impulsivity problems. However, the faster rate of progression to pathological gambling and drug addiction seen in females as compared to males suggests a strong vulnerability to impulsivity problems among a subset of the female population. These differences in prevalence rates and course underscore the complexity of the many facets of impulsivity.

#### *Sex differences in impulsivity in the general population*

Sex differences in the anatomy and neurochemistry of brain regions involved in impulsive action and impulsive choice do exist. For example, both post-mortem and imaging studies have found that relative to brain size, women have larger volumes in the striatum (Filipek et al. 1994), ACC (Paus et al. 1996), and dorsolateral PFC

(Schlaepfer et al. 1995). Several studies show that the availability of dopamine transporters, which regulate synaptic dopamine levels, is significantly greater in women than in men (Lavalaye et al. 2000; Mozley et al. 2001). Similarly, in the striatum, female presynaptic dopamine levels exceed those of age-matched males (Laakso et al. 2002). In addition, GABA levels are higher in women than in men throughout the cortex (Sanacora et al. 1999).

Only a small number of laboratory studies have examined sex differences in impulsivity in the general population. With regard to impulsive action in humans, behavioral performance on the Stroop task and stop signal task does not seem to differ between the sexes (Bolla et al. 2004; Huster et al. 2011). On other measures of risk taking or impulsivity, mixed reports have shown males to be more impulsive (Kirby and Marakovic 1996; Rosenblitt et al. 2001; Van et al. 2008; Whiteside and Lynam 2003), less impulsive (Reynolds et al. 2006) or equally impulsive (Fillmore and Weafer 2004; Skinner et al. 2004) compared to females. Experimental conditions seem to play an important role in these mixed results, possibly because males and females may use different strategies on the various tasks employed. For example, women show greater impulsive choice than men when rewards are hypothetical, but when rewards are real, men show greater impulsive choice than women (Heyman and Gibb 2006). Another reason for the mixed findings might be that sex differences in impulsivity may be developmental in nature and are most detectable prior to the full maturation of the PFC. There are some reports of sex differences in impulsive choice in children. For example, delayed gratification scores are higher for girls than for boys when asked to

withhold from eating candy until instructed to do so by an interviewer (Li-Grining 2007). In addition, girls are rated by their parents as displaying greater inhibitory control than are boys on an inhibitory control questionnaire (Moilanen et al. 2009).

Impaired planning and decision-making is a component of impulsivity, particularly impulsive choice. Studies examining decision-making have revealed sex differences (Bolla et al. 2004; Tranel et al. 2002). Men outperform women on the Iowa Gambling Task (Overman 2004; Reavis and Overman 2001). This task requires participants to choose cards from two decks that result in either high rewards with infrequent but high losses (the disadvantageous deck) or low rewards with more frequent but low losses (the advantageous deck). While control participants learn to choose the advantageous deck, patients with PFC damage (Bechara et al. 1994), drug abusers (Grant et al. 2000), and pathological gamblers (Cavedini et al. 2002) fail to do so. Interestingly, men choose cards from the advantageous deck more often than do females (Crone et al. 2005; Kerr and Zelazo 2004; Overman 2004; Reavis and Overman 2001). It is believed that there is a sex difference in the strategy employed to solve the task (Overman 2004). While men learn to maximize monetary gains in the long run by choosing only cards from the advantageous deck, females tend to choose cards from decks that have the lowest frequency of loss, even if that loss is very large as it is with the disadvantageous deck. During performance of the Iowa Gambling Task brain activation is more lateralized to the right hemisphere in men, while females show activation in both hemispheres (Bolla et al. 2004), supporting the possible use of different strategies by the sexes.

## **Animal Models of Impulsivity**

Impulse control can be assessed in animals using procedures that have been developed to model particular aspects of impulsivity. Impulsive action is commonly measured in rodents using the 5-choice serial reaction time task (5-CSRTT), during which animals must suppress the impulse to make a response until the appropriate time (Eagle and Baunez 2010; Harrison et al. 1999). The 5-CSRTT was developed from the continuous performance task used to quantify attentional and impulsive deficits in humans (Robbins 2002). In the 5-CSRTT, rats must identify the location of a brief light stimulus presented randomly across five possible locations over a large number of independent trials by making a nose poke into the previously light aperture (Carli et al. 1983). After baseline training, the time before the onset of the light stimulus can be lengthened to challenge inhibitory control and assess impulsive action. Impulsive choice is commonly measured in rodents using the delay-based impulsive choice task where animals are given the choice between a small food reward and a large food reward (Eagle and Baunez 2010). To challenge and assess impulsive choice, delays are successively added to the large food reward but not to the small food reward (Rudebeck et al. 2006). Impulsive choice is defined as the selection of the immediate small food reward over the delayed large food reward (Dalley et al. 2008).

### *Sex differences in animal models of impulsivity*

Compared to the number of studies researching impulsivity in male rodents, few studies have investigated impulsivity in female rodents. These studies examining sex

differences in impulsivity have yielded mixed results. Adult male rats have been reported to make more impulsive actions than do adult female rats during a test of spatial divided attention (Jentsch and Taylor 2003). However, another study reported no sex difference in impulsive action in adult rats (Burton and Fletcher 2012). Male rats have also been reported to display increased impulsive novelty-seeking behavior compared to female rats at mid-adolescence (pnd 40) but not at early-adolescence (pnd 28) or early adulthood (pnd 80) (Cyrenne and Brown 2011). Sex differences have also been reported in impulsive choice behavior during an operant delay-discounting task. Under strong food deprivation male mice made more impulsive choices than did female mice, yet under mild food deprivation female mice made more impulsive choices than did male mice (Koot et al. 2009), indicating that factors such as stress, motivation, and age may contribute to sex differences in impulsivity.

#### *Neonatal hormones and sex differences*

The magnitude of sex differences in the size of brain structures showing sexual dimorphisms correlates with the degree to which the regions express sex steroid receptors during development (Goldstein et al. 2001). This suggests that structural differences are at least partially mediated by developmental levels of sex hormones. Neonatal sex hormone levels alter brain development (Gorski 1993). Sexual differentiation in mammals begins with the expression of the SRY gene on the Y chromosome in XY males, which drives the formation of testes from the bipotential gonads (McCarthy 1994). The testes produce the anti-Mullerian hormone (AMH), which

inhibits the development of ovaries and accessory female reproductive tissues. During the critical neonatal period of development in rodents, testosterone produced by the testes circulates throughout the body and freely enters the brain. In contrast, the female rodent brain is relatively unaffected by sex steroids due to their quiescent ovaries and high levels of circulating  $\alpha$ -fetoprotein, which is produced by the embryo and binds to maternal estrogens preventing them from entering the brain (Rhoda et al. 1984). Phoenix et al. (1959) were the first to demonstrate that prenatal testosterone masculinizes and defeminizes behaviors in rodents resulting in an increase in male-typical behaviors and a decrease in female-typical behaviors. In the absence of the SRY gene and AMH during the critical neonatal period of development, sexual differentiation in XX females proceeds in an inherently female direction with the development of ovaries and female behavioral characteristics (Nelson 2005a). In many brain areas, such as the hypothalamus, the effects of neonatal testosterone on brain development are dependent upon its conversion into estradiol by the enzyme aromatase once inside the cell (Gorski 1993). The mechanism of action of neonatal testosterone in some brain areas, such as the PFC, is understudied and unknown.

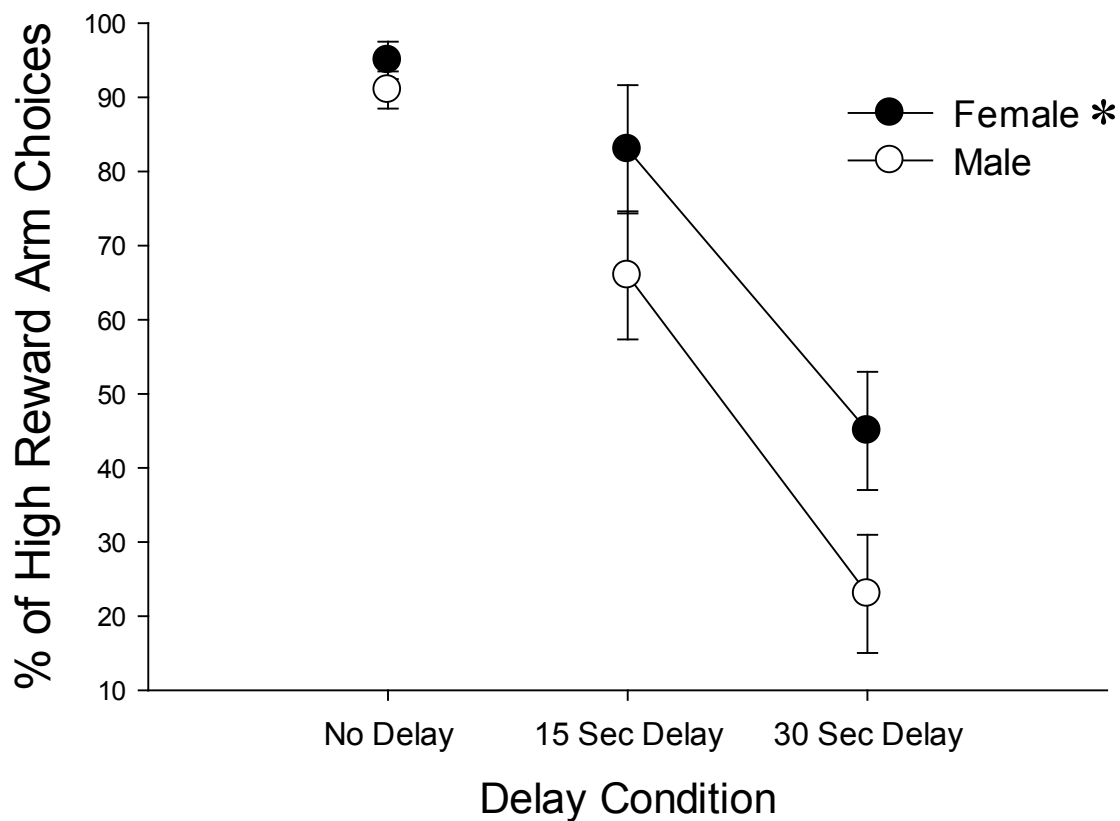
Since the publication of the Phoenix et al. (1959) paper, many behaviors in a wide range of animals have been shown to be influenced by the organizing effects of neonatal sex hormone levels (Arnold 2009; Gorski 1993; Williams and Meck 1991). For example, female rats exposed to neonatal testosterone display enhanced male-like performance and male-like strategy use in solving the radial arm maze (Roof 1993). In addition, exposure to neonatal testosterone increases male-like mounting behavior,

rough play, and vocalizations in female rhesus monkeys (Thornton et al. 2009). Similar effects are seen in women exposed to higher than normal levels of testosterone during development. Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder that causes elevated prenatal levels of adrenal androgens, particularly testosterone (Hines 2006). Girls with CAH typically show elevated levels of male-typical behavior such as increased boy-typical childhood play (Berenbaum et al. 2000), higher levels of aggression (Berenbaum and Resnick 1997), and higher masculinity scores on questionnaires (Hall et al. 2004).

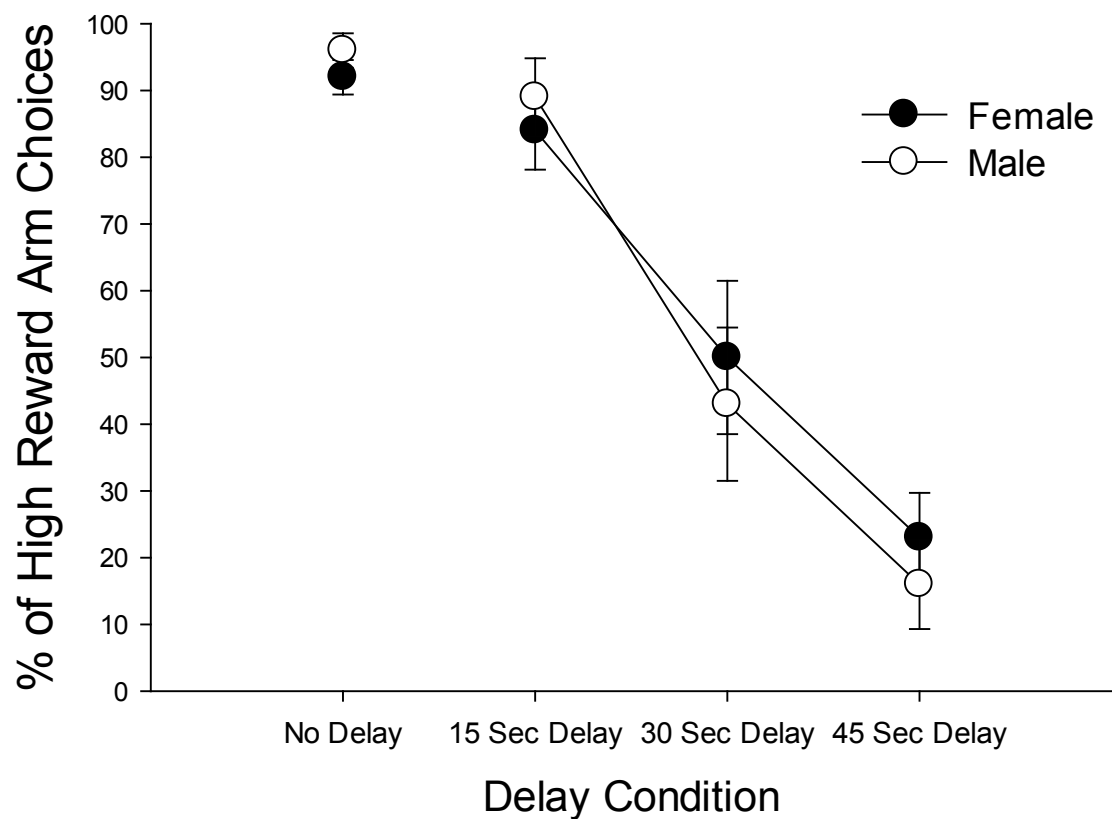
### **Preliminary Studies**

Previous research in our laboratory has explored sex differences and the role of neonatal testosterone exposure on impulsive choice behavior in male and female rats using the delay-based impulsive choice task. To examine sex differences before and after puberty, male and female prepubertal and adult rats were tested on the task. Prepubertal male rats made more impulsive choices than did prepubertal female rats (see Figure 1). This difference was not observed in adult rats (see Figure 2). To examine the role of neonatal testosterone exposure, male and female rats were treated with either testosterone propionate or vehicle on days 1 and 2 post birth and tested on the task before puberty. Like prepubertal male rats, prepubertal female rats exposed to neonatal testosterone made more impulsive choices than did control prepubertal female rats (see Figure 3). These results indicate that the increased impulsivity displayed by prepubertal male rats is mediated by neonatal testosterone exposure.

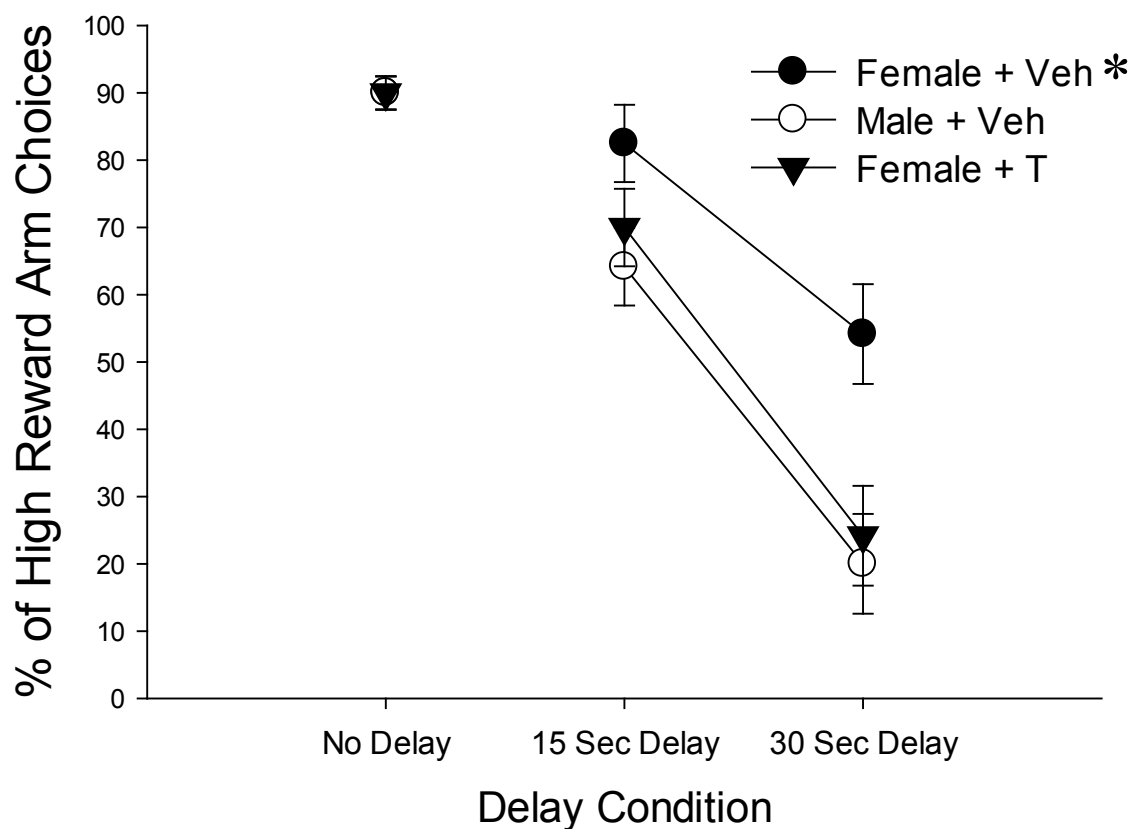
Figure 1



**Figure 1.** Effect of increasing delay for high reward (HR) on percentage of HR arm choices in male and female prepubertal rats. Males made significantly fewer HR arm choices than did females when delays were imposed,  $*p < .05$  vs. Male. (Bayless et al. 2013)

**Figure 2**

**Figure 2.** Effect of increasing delay for high reward (HR) on percentage of HR arm choices in male and female adult rats. Males and females were not significantly different in terms of HR arm choices.

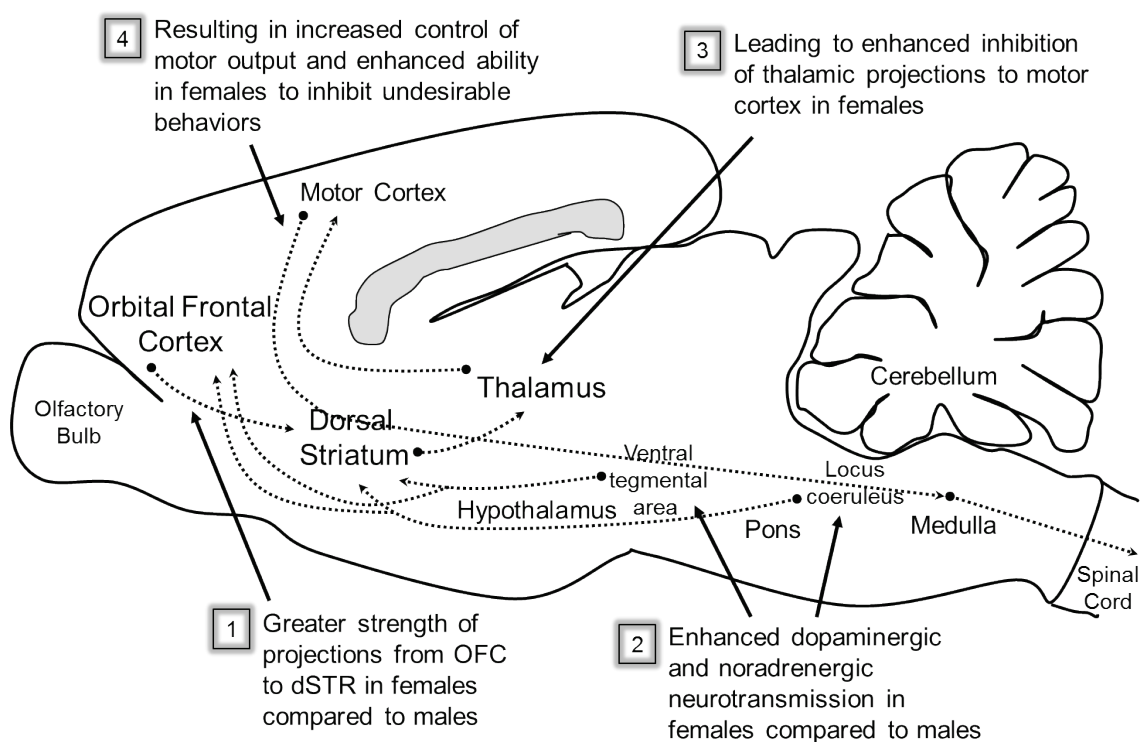
**Figure 3**

**Figure 3.** Effect of increasing delay for high reward (HR) on percentage of HR arm choices in testosterone-treated female (Female + T), control female (Female + Veh), and control male (Male + Veh) prepubertal rats. Neonatal testosterone-treated females and control males made significantly fewer HR arm choices than did control females across delays,  $*p < .05$  vs. Male + Veh and Female + T. (Bayless et al. 2013)

## Aims and Hypotheses

The broad objective of the current research was to test the hypothesis that there is a sex difference in impulsivity in prepubertal and adult rats, such that female rats have greater impulse control than do male rats and that this increased impulse control may be the result of greater dopaminergic and noradrenergic activity and enhanced OFC projections to and control over the dSTR in females as compared to males, thereby providing females with a greater ability than males to inhibit undesirable behaviors (Figure 4).

**Figure 4**



**Figure 4.** Proposed model of molecular sex differences contributing to the increased impulse control in females as compared to males. OFC: orbital frontal cortex; dSTR: dorsal striatum

The current research focused on four aims. The first aim was to determine the mechanism by which neonatal testosterone exerts its masculinizing effects on impulsive behavior. The second aim was to investigate and compare sex differences in impulsive choice and impulsive action in adult rats. The third aim was to identify possible sex differences in dopaminergic and noradrenergic functioning in the OFC and dSTR, two brain areas and neurotransmitter systems involved in impulsivity as previously discussed. The fourth aim was to examine if greater OFC control over the dSTR in females as compared to males provides females with a greater ability than males to inhibit undesirable behaviors.

To complete Aim 1, I conducted an experiment to determine the mechanism by which neonatal testosterone exerts its masculinizing effects on impulsive behavior. In order to avoid adult activational levels of hormones and isolate organizational effects, I studied prepubertal rats. Preliminary studies demonstrated that neonatal testosterone exposure can masculinize impulsive choice behavior of prepubertal female rats. Because testosterone can be aromatized into estradiol, this organizational effect could result from neonatal activation of either androgen or estrogen receptors. To determine if activation of androgen receptors or activation of estrogen receptors via the conversion of testosterone into estradiol mediates the sex difference in impulsive choice behavior, I treated prepubertal rats with neonatal hormones activating either estrogen or androgen receptors and tested the animals on the delay-based impulsive choice task prior to puberty. The mechanism of action of neonatal testosterone in the PFC is unknown.

*Based on the action of neonatal testosterone in the hypothalamus, I hypothesized that*

*neonatal activation of estrogen receptors but not androgen receptors would masculinize impulsive choice behavior in prepubertal female rats.*

To complete Aim 2, I conducted experiments to investigate and compare sex differences in both impulsive choice and impulsive action in adult rats. Given the short time window before puberty in the rat, the investigation of the role of OFC control over the dSTR in impulsivity is better suited for study in adult rats. However, in order to study the role of OFC control over the dSTR in impulse control, a sex difference in impulsive choice or impulsive action in adult animals must first be established. Preliminary studies indicated that there is no sex difference in impulsive choice responding in adult rats. However, it was unclear whether or not the delays used in the study were long enough to reliably measure a difference in performance in adults, which were not as affected by the delay lengths used in prepubertal rats. In Experiment 2a, I used increased delays to determine if a sex difference in impulsive choice behavior existed in adult rats during performance of the delay-based impulsive choice task. *I hypothesized that when the demands on impulse control were increased, a sex difference in impulsive choice responding would be discovered, such that adult female rats would make fewer impulsive choices than would adult male rats.*

In Experiment 2b, I set out to determine if a sex difference in impulsive action behavior existed by testing adult male and female rats on the 5-CSRTT. Because of the length of training required for the 5-CSRTT and similar impulsive action tasks, impulsive action was not able to be measured in prepubertal rats. *I hypothesized that similar to*

*impulsive choice responding, adult female rats would make fewer impulsive actions than would adult male rats.*

To complete Aim 3, I conducted experiments to examine possible neurochemical differences between males and females that might contribute to the sex differences previously reported in impulsive choice behavior in prepubertal rats. In Experiment 3a, using the brain tissue from the prepubertal control male and female rats and female rats treated with neonatal testosterone from the preliminary studies, I looked for sex differences in markers of dopaminergic and noradrenergic reuptake in the OFC and dSTR. To assess dopamine and norepinephrine levels, I conducted western blots on the transporters for these neurotransmitters, DAT and NET respectively. Increased levels of dopamine and norepinephrine result in the up-regulation of their respective transporters (Furman et al. 2009; Sager and Torres 2011). Therefore, increased transporter levels are indicative of increased signaling of the neurotransmitters that they flux. In addition, drugs such as methylphenidate and atomoxetine, which effectively reduce impulsive behavior in humans and animals, act at these transporters (Marchant et al. 2011; Rucklidge 2010). As discussed above, high levels of norepinephrine in the OFC are associated with increased inhibitory control. Mixed results in terms of the direction of effects are reported for dopamine. *I hypothesized that control prepubertal female rats would have increased levels of DAT and NET in the OFC and the dSTR as compared to control male rats and female rats treated with neonatal testosterone, indicating greater dopaminergic and noradrenergic neurotransmission, which could contribute to increased inhibitory control.*

In Experiment 3b, using the brain tissue of the adult male and female rats from the preliminary studies, I looked for sex differences in markers of dopaminergic and noradrenergic reuptake in the OFC and dSTR by conducting western blots on DAT and NET. Although no sex difference was found in impulsive choice behavior in adult rats in the preliminary studies, based upon the results I inferred that in order to conclusively rule out a sex difference in adult animals further testing was needed as was discussed above. *I hypothesized that adult female rats would have increased levels of DAT and NET in the OFC and the dSTR as compared to adult male rats, indicating greater dopaminergic and noradrenergic neurotransmission, which could contribute to increased inhibitory control.*

To complete Aim 4, I conducted experiments to explore the role of OFC control over the dSTR in impulsive action. In Experiment 4ai and 4aii, I examined sex differences in the levels of OFC and dSTR myelination by conducting western blots on the same prepubertal and adult brain tissue used in Experiments 2a and 2b for two markers of myelination, myelin basic protein (MBP) and myelin proteolipid protein (PLP). PLP is a hydrophobic integral membrane protein and the most abundant protein in CNS myelin (Greer and Lees 2002). MBP is the second most abundant protein in CNS myelin and is responsible for the adhesion of multilayered compact myelin to axons and to itself (Boggs 2006). MBP consists of four major isoforms with molecular masses of 21.5, 18.5, 17.0, and 14.0 kDa (Akiyama et al. 2002). Increased levels of MBP and PLP are indicative of increased myelin levels. Increased myelin levels indicate either an increase in neuronal connections or an increase in axonal diameter of neurons. Both the number of

neuronal connections and axonal diameter are associated with faster, more efficient neuronal communication (Sherman and Brophy 2005). *I hypothesized that prepubertal and adult females would have increased levels of MBP and PLP in the OFC but not the dSTR as compared to males, indicating that neurotransmission is faster in the OFC but similar in the dSTR in females as compared to males perhaps leading to increased OFC communication with and control over the dSTR in females as compared to males.*

In Experiment 4b, I set out to examine if a sex difference in the strength of projections from the OFC to the dSTR existed by infusing an anterograde tracer into the OFC and measuring the levels of tracer in the dSTR. *I hypothesized that when an anterograde tracer was taken up by neurons in the OFC, females would have a greater expression of the tracer in the dSTR than would males, indicating that females have more projections from the OFC to the dSTR than do males.*

In Experiment 4c, I tested the hypothesis that decreased control of the OFC over the dSTR would lead to increased impulsive action by using a simplified version of the 5-CSRTT and inactivating the OFC in adult animals. The task was simplified with only one stimulus location to isolate impulsive action and focus on the inhibition of a habitual striatal response. *I hypothesized that if a sex difference in impulsive action is the result of females having greater OFC control over the dSTR than males, then the inactivation of the OFC should decrease female impulsive action levels to that of males, eliminating the sex difference in impulsive action.*

Taken together, these studies determined the mechanism by which neonatal testosterone exerts its masculinizing effects on impulsive choice behavior, investigated and compared possible sex differences in impulsive choice behavior and impulsive action behavior in adult rats, identified possible sex differences in dopaminergic and noradrenergic functioning in the OFC and dSTR, and elucidated the role of increased OFC control over the dSTR as a possible molecular sex difference that could provide females with enhanced impulse control as compared to males.

**Aim 1: To determine the mechanism by which neonatal testosterone exerts its  
masculinizing effects on impulsive behavior**

**Experiment 1: Effect of neonatal activation of estrogen and androgen receptors on the  
delay-based impulsive choice task in prepubertal rats**

*Hypothesis*

Previous research in our laboratory has demonstrated that neonatal testosterone exposure masculinizes impulsive choice behavior in prepubertal female rats. Because testosterone can be aromatized into estradiol, the organizational effect of neonatal testosterone could have resulted from neonatal activation of either androgen or estrogen receptors. I hypothesized that neonatal activation of estrogen receptors but not androgen receptors would masculinize impulsive choice behavior in prepubertal female rats.

**Methods**

*Subjects*

Twenty-four female Long-Evans hooded rats and sixteen male Long-Evans hooded rats were obtained from litters bred in the lab. In order to breed these animals, nine female Long-Evans hooded rats and nine male Long-Evans hooded rats, approximately 55 days of age, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) to be used as breeders. Animal care was in accordance with the guidelines set by the *National Institutes of Health Guide for the Care and Use of*

*Laboratory Animals* and all procedures were approved by the Institutional Animal Care and Use Committee of Tulane University. Rats were group housed with two to three rats of the same sex and treatment group per cage in a temperature controlled vivarium under a 12-h light/dark cycle (lights on at 7:00 a.m.). All testing was completed before rats reach 35 days of age and before the onset of puberty. The onset of puberty is typically around 35 days of age for female rats and 45 days of age for male rats (Kennedy and Mitra 1963).

### *Breeding*

In order to facilitate behavioral testing, testing was conducted in three replicates obtained by breeding three female Long-Evans hooded rats with three male Long-Evans hooded rats for each replicate. Females were mated and allowed to deliver normally 22-23 days after conception. Litters were culled to 5 males and 5 females when possible to reduce variability in maternal care. Rats were weaned at 21 days of age and group housed by sex and treatment condition. Behavioral testing began at 24 days of age. Two male and three female rats were used from each litter and remaining rats were used in other experiments or sacrificed at weaning.

### *Hormone manipulation*

On the day of birth and 24 h later, eight female pups were treated with 150  $\mu$ g of estradiol benzoate (activating only estrogen receptors) delivered in 0.1 ml of sesame oil vehicle, eight female pups were treated with 150  $\mu$ g of the non-aromatizable androgen dihydrotestosterone benzoate (activating only androgen receptors) delivered in 0.1 ml of sesame oil vehicle, and eight male pups were treated with 150  $\mu$ g of the aromatase

inhibitor formestane, which blocks the conversion of testosterone to estradiol (activating only androgen receptors), delivered in 0.1 ml of sesame oil vehicle (See Amateau et al. 2004; Mitsushima et al. 2009; Zhang et al. 2008). To serve as controls, eight female pups and eight male pups received parallel injections of 0.1 ml of sesame oil vehicle. Injections were made subcutaneously on the dorsal flank of each rat. Skin glue was applied to seal the injection site.

#### *Verification of hormone treatment*

Neonatal testosterone treatment increases the anogenital distance of females to be closer to that of male rats (Welsch et al. 2009). To verify the efficacy of the hormone treatment, anogenital distance was measured under anesthesia prior to euthanasia at 35 days of age.

#### *Apparatus*

Behavioral testing on the delay-based impulsive choice task was conducted in a plastic T-maze (arms: 10 cm wide x 40 cm long x 20 cm high). The floor of the maze was made of solid black plastic, and the walls of the maze were made of clear plastic. The start arm (Winstanley et al. 2004) led to two goal arms (east and west, respectively). A clear plastic 25-cm-high sliding door was placed 5 cm into the entrance of each goal arm designed to confine the rat into that goal arm upon entry. A second clear plastic 25-cm-high sliding door was placed 5 cm from the end wall of each goal arm designed to impose a delay between the arm choice and food access.

### *Habituation*

Rats were placed on food-restricted diets and weighed daily throughout the experiment to maintain their body weights at approximately 90% of the average free feeding weight for aged-matched Long-Evans rats according to a standard growth chart (Harlan). At 24 days of age, cage mates were placed into the maze for two separate 15-min acclimation periods during which an equal amount of Froot Loops was placed at the end of each goal arm. All four sliding doors were removed, and the rats were allowed to travel freely throughout the maze. Several Froot Loops were placed in the home cages each day.

### *No-delay trials*

Beginning the day after habituation at 25 days of age, rats were trained to choose between a low-reward (LR) arm that contained one piece of Froot Loop and a high-reward (HR) arm that contained five pieces of Froot Loop. Rats were trained and tested in assigned pairs counterbalanced across all treatment groups in which rats alternated trials each session. The location of the HR arm was counterbalanced across pairs. The maze was cleaned with ethanol between trials. To ensure the completion of all behavioral testing before the onset of puberty, rats were given one to two sessions each day. Each session started with a forced trial into each of the LR and HR arms during which a black plastic sliding door blocked access to the opposite arm. The order of these forced trials alternated each session. Following the forced trials, rats received five choice trials in which they were free to choose either the LR or HR arms. When a rat entered an arm the first sliding door was lowered to confine the rat in the arm. The

second sliding door was then lifted to give the rat immediate access to the food reward. Sessions of this no-delay condition continued until rats were choosing the HR arm on at least 80% of the trials for two consecutive sessions.

#### *Delay trials*

After the no-delay sessions at 30 days of age, rats received three 15-sec delay sessions. The first session of each delay was given to habituate and expose the rats to the delay conditions. Performance during the final two sessions was used for analysis. During these sessions, a 15-sec delay was imposed when rats entered the HR arm. The 15-sec delay on the HR arm was imposed during the two forced trials and all five choice trials. When a rat entered the HR arm, the first sliding door was lowered to confine the rat in the arm. The rat then had to wait 15 sec before the second sliding door was lifted to provide access to the larger food reward. When a rat entered the LR arm, the first sliding door was lowered to confine the rat in the arm and the second sliding door was lifted to provide immediate access to the smaller food reward. After the three 15-sec delay sessions at 33 days of age, rats received three 30-sec delay sessions. The procedure during these sessions was the same as in the 15-sec delay sessions except that the delay on the HR arm was increased to 30 sec.

#### *Verification of prepubescence*

Puberty in female rats is marked by vaginal canalization (Kennedy and Mitra 1963). To verify that the onset of puberty had not occurred in female rats, vaginal opening was visually examined in female rats the day after the completion of testing.

### *Statistical analyses*

The percentages of HR arm choices during the choice trials from the final two sessions of the no delay condition were averaged and analyzed using a one-way ANOVA. The percentages of HR arm choices during the choice trials from the final two sessions of each delay were averaged and analyzed using a repeated measures ANOVAs with delay as the within-subjects factor and hormone treatment as the between-subjects factor. Anogenital distances were analyzed using a one-way ANOVA. When a significant main effect of hormone treatment was discovered, Fisher's LSD pair-wise comparison procedures were conducted.

## **Results**

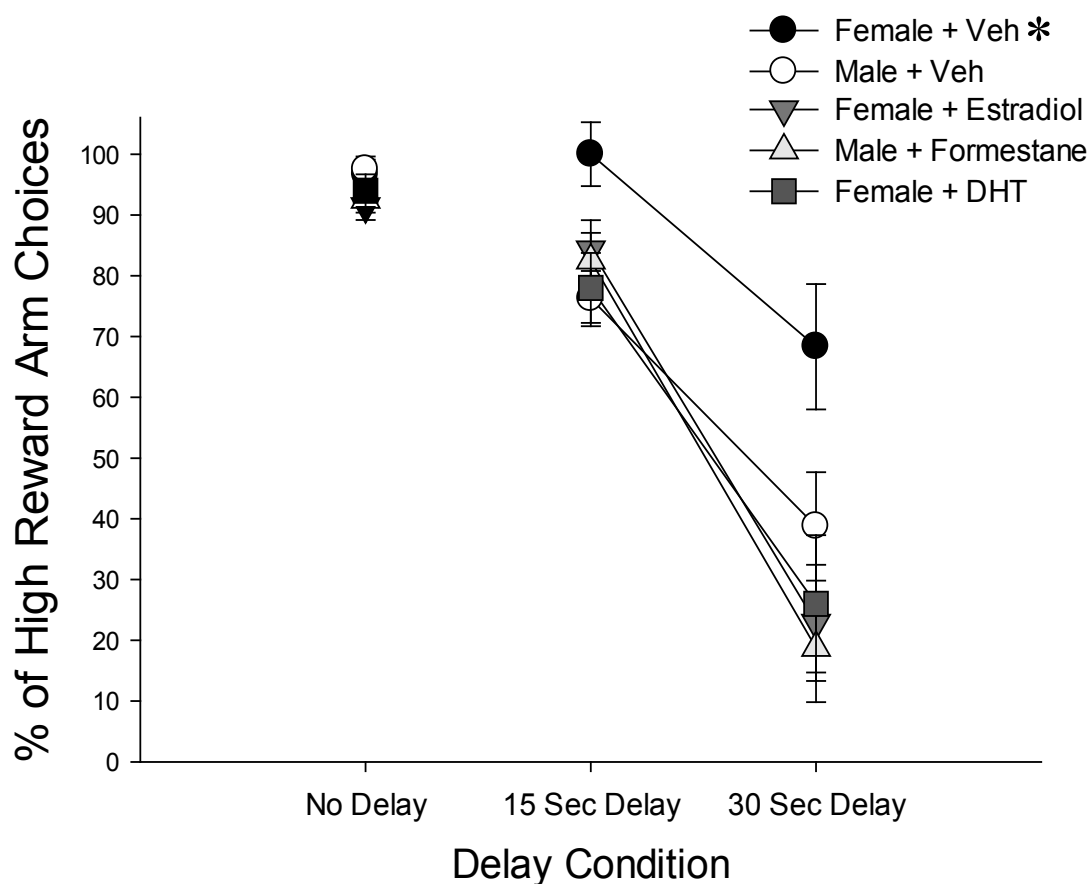
### *Performance on the delay-based impulsive choice task*

As illustrated in Figure 5, estradiol-treated females, DHT-treated females, and formestane-treated males displayed increased impulsivity as compared to control females and their performance was indistinguishable from that of control males. Analysis of the percentages of HR arm choices from the final two sessions of the no-delay condition revealed no significant main effect of hormone treatment, indicating that rats in all groups equally preferred the larger reward over the smaller reward when rewards were immediately available. Analysis of the percentages of HR arm choices across the 15-sec and 30-sec delay conditions revealed a significant main effect of hormone treatment,  $F(4, 29) = 4.51, p = .006$ . As follow-up to the significant main effect of hormone treatment, Fisher's LSD pair-wise comparisons revealed that estradiol-

treated females (estrogenic effect;  $p = .002$ ), DHT-treated females (androgenic effect;  $p = .003$ ), formestane-treated males (androgenic effect;  $p = .001$ ), and control males ( $p = .005$ ) all made significantly fewer HR arm choices than did control female rats when delays were imposed before access was allowed to the high reward.. There was also a significant main effect of delay,  $F(1, 29) = 148.66$ ,  $p < .001$ , indicating that choice of the HR arm decreased with the increasing delay for each treatment group. There was no significant interaction between treatment and delay.

Analysis of anogenital distance revealed a significant main effect of hormone treatment,  $F(4, 29) = 108.687$ ,  $p < .001$ . As follow-up to the significant main effect of hormone treatment, Fisher's LSD pair-wise comparisons revealed that the anogenital distance of control female rats (mean  $\pm$  SEM;  $9.67 \text{ mm} \pm .525$ ), DHT-treated female rats ( $10.60 \text{ mm} \pm .576$ ), and estradiol-treated female rats ( $11.00 \text{ mm} \pm .486$ ) were significantly shorter ( $p < .001$ ) than aromatase inhibitor-treated male rats ( $19.50 \text{ mm} \pm .455$ ) and control male rats ( $19.75 \text{ mm} \pm .455$ ). Unexpectedly the anogenital distance of DHT-treated females was not significantly increased in the male direction.

Figure 5



**Figure 5.** Effect of increasing delay for high reward (HR) on percentage of HR arm choices in neonatal estradiol-treated female (Female + Estradiol), neonatal-aromatase inhibitor, formestane-treated male (Male + Formestane), neonatal dihydrotestosterone-treated female (Female +DHT), control female (Female + Veh), and control male (Male + Veh) prepubertal rats. Neonatal estradiol-treated females, formestane-treated males, DHT-treated females, and control males made significantly fewer HR arm choices than did control females across delays,  $*p < .05$  vs. all other groups. (Bayless et al. 2013)

**Aim 2: To investigate and compare sex differences in impulsive choice and impulsive action in adult rats**

**Experiment 2: Performance of adult male and female rats on tasks of impulsive choice and impulsive action**

**Experiment 2a: Performance of adult male and female rats on the delay-based impulsive choice task under increased delay conditions**

*Hypothesis*

Preliminary studies indicated that there was no sex difference in impulsive choice responding in adult rats. However, it was unclear whether or not the delays used in the study were long enough to reliably measure a difference in performance in adults, which were not as affected by the delay lengths used in prepubertal rats. I hypothesized that when demands on impulse control were increased, a sex difference in impulsive choice responding would be discovered, such that adult female rats would make fewer impulsive choices than would adult male rats.

**Methods**

*Subjects*

Six female Long-Evans hooded rats and six male Long-Evans hooded rats, approximately 70 days of age, were purchased from Harlan Sprague-Dawley. Rats were housed in same-sex pairs, and animal care was the same as described in Experiment 1.

All animals were weighed daily following behavioral testing and food was provided in their home cages to maintain their weights at 85% of their free-feeding weights while allowing for growth of approximately 2% of their body weight each week.

### *Behavioral testing*

Testing procedures and statistical analyses were the same as described in Experiment 1, except that delays of 20, 40, and 60 secs were imposed on the HR arm to increase the difficulty for adult rats.

### *Vaginal cytology*

To assess that all female rats were displaying normal estrous cycles, vaginal smears of female rats were collected by lavage each morning and analyzed daily beginning two weeks prior to behavioral testing. To control for handling effects, males were given sham smears during which a small amount of water was placed on the genitals using a medicine dropper.

### *Statistical analyses*

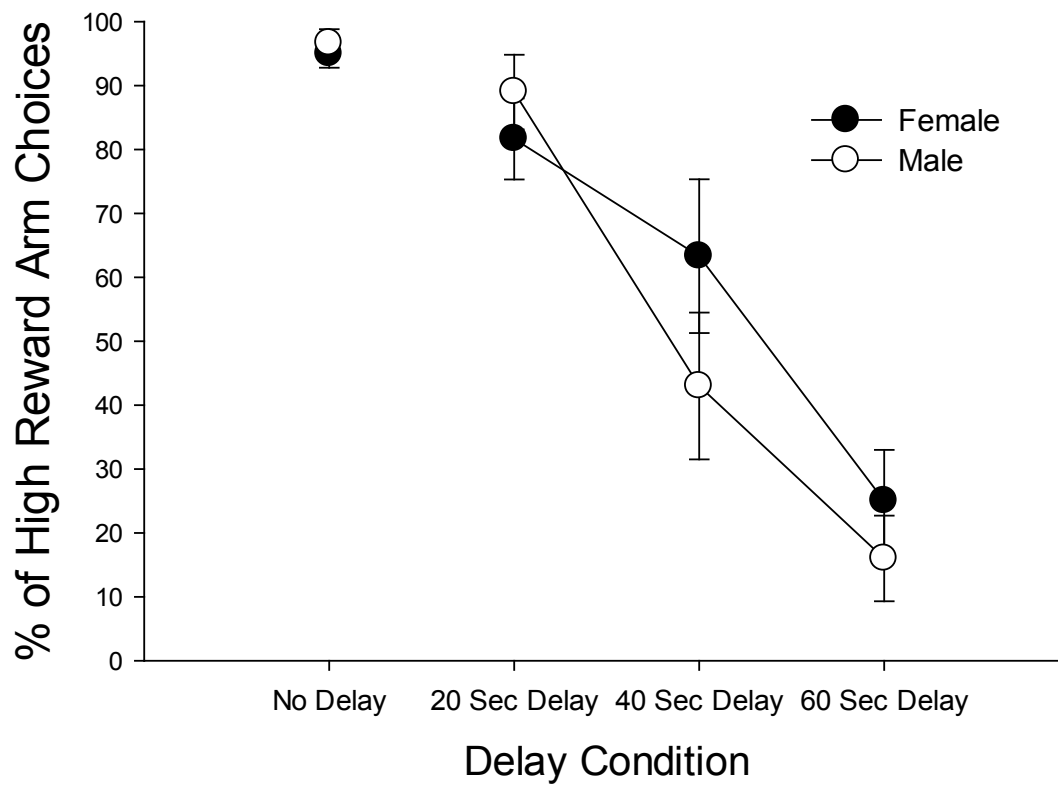
The percentages of HR arm choices during the choice trials from the final two sessions of the no delay condition were averaged and analyzed using a one-way ANOVA. The percentages of HR arm choices during the choice trials from the final two sessions of each delay were averaged and analyzed using a repeated measures ANOVAs with delay as the within-subjects factor and sex as the between-subjects factor. To examine female performance across the estrous cycle, the percentages of HR arm choices from each delay session were analyzed using a repeated measures ANOVAs with delay

session as the within-subjects factor and estrous cycle stage as the between-subjects factor.

## Results

### *Performance on the delay-based impulsive choice task*

As illustrated in Figure 6, male and female adult rats displayed similar levels of impulsive choice behavior, as measured by performance on the delay-based impulsive choice task. Analysis of the percentages of HR arm choices from the final two sessions of the no delay condition revealed no significant main effect of sex, indicating that male and female rats equally preferred the larger reward over the smaller reward when rewards were immediately available. Analysis of the percentages of HR arm choices across delay condition revealed no significant main effect of sex, indicating that adult male and female rats did not differ in terms of number of HR arm choices. There was a significant main effect of delay,  $F(2, 20) = 30.61$ ,  $p < .001$ , indicating that choice of the HR arm decreased with the increasing delay for each sex. There was no significant interaction between sex and delay. In addition, there was no main effect of estrous cycle stage, delay session, or interaction between estrous cycle stage and delay session in females, indicating that impulsive choice behavior did not vary across the stages of the estrous cycle.

**Figure 6**

**Figure 6.** Effect of increasing delay for high reward (HR) on percentage of HR arm choices in adult male and female rats. Males and females were not significantly different in terms of HR arm choices.

## **Experiment 2b: Performance of adult male and female rats on the 5-CSRTT, a test of impulsive action**

### *Hypothesis*

I hypothesized that adult female rats would make fewer impulsive actions than would adult male rats.

## **Methods**

### *Subjects*

Fourteen male and fourteen female Long-Evans hooded rats, approximately 2 months of age, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed in same-sex pairs, and animal care was the same as described in Experiment 1. All animals were weighed daily following behavioral training and food was provided in their home cages to maintain their weights at 85% of their free-feeding weights while allowing for growth of approximately 2% of their body weight each week.

### *Apparatus*

Animals were trained and tested in one of four separate 25x25 cm aluminum chambers (Lafayette Instrument Co., Lafayette, IN). The rear wall of each chamber was convexly curved and contained five apertures, each 2.5 cm square, 4 cm deep, and set 2 cm above floor level. Each hole could be illuminated with a 3 W light bulb located at the rear of the hole. Each hole had an infrared photocell beam monitoring the entrance. The four conditioning chambers were individually housed in sound attenuating cabinets. Each chamber was illuminated by a 3 W house light and equipped with a speaker that

could deliver bursts of white noise. The front wall could be opened to place in and remove the animal from the chamber. On the front wall, 25 cm from each nose-poke hole, there was a food magazine where 45 mg food pellets (Test Diet, Richmond, IN) could be automatically dispensed. Each animal received one session of training per day throughout the experiment. House lights were on unless stated otherwise.

### *Behavioral training*

First, animals were successively shaped to retrieve food rewards from the food tray and to poke any of the holes to receive food rewards. Then each animal was trained daily for 30 min on the 5-choice serial reaction time task (5-CSRTT) by passing through several training stages of increasing difficulty. Each session was terminated after 100 trials had been completed or 30 min had expired, whichever occurred first. An animal was moved to the next training stage once it performed 100 trials at >80 percent correct and <20 percent omission for two consecutive days. Percent correct reflected the percentage of correct responses, whereas percent omission reflected the failure to respond to the stimulus. Each rat was always trained in the same conditioning chamber. Females were always trained in the same two chambers while males were always trained at the same time as the females in the other two chambers. Animals were trained at approximately the same time of the light phase each day.

For the initial training stage, animals were placed in the chamber and could initiate the first trial by retrieving a single food pellet from the food tray. After a fixed 5 sec inter-trial interval (ITI), one of the five horizontal lights would illuminate for a maximum of 60 sec (cue duration) or until a response had been made. From the time

the light first turned on, the animal had 60 sec (limited hold period) to respond by making a nose poke into the previously lit aperture. Correct responses were immediately rewarded with delivery of a food pellet into the food magazine, and retrieval of the food restarted the next trial after a 5 sec ITI. Several types of errors were recorded: i) Nose pokes during the ITI were recorded as premature responses; ii) Repeated nose pokes into the correct aperture were recorded as perseverative responses; iii) Responding into a non-lit aperture was recorded as an incorrect response; iv) Failure to respond within the limited hold period was recorded as an omission. All errors were punished by switching off the house light for a 5 sec time-out period, and no food was delivered. Responses to holes during this period would restart the time-out period.

For subsequent training stages all parameters remained the same, but the stimulus duration was successively decreased from 60 sec to 0.6 sec and the limited hold period was successively decreased from 60 sec to 5 sec. For the final training stage (baseline training), the cue duration was further reduced to 0.5 sec. Training with this protocol continued until animals perform 100 trials at a criterion of >70 percent correct with <20 percent omissions for five consecutive days.

#### *Vaginal cytology*

To control for effects of fluctuating ovarian hormones on performance, vaginal smears of female rats were collected by lavage each morning and analyzed daily beginning two weeks prior to behavioral testing. To control for handling effects, males were given sham smears during which a small amount of water was placed on the

genitals using a medicine dropper. Behavioral challenge conditions were only administered when a female was at the proestrous stage of the estrous cycle, at which time estradiol levels are at their peak and vaginal cytology is characterized by large nucleated epithelial cells (Becker et al. 2005). Each male was paired with a particular female and was always tested at the same time as that female.

### *Behavioral testing*

The following series of manipulations to challenge performance were introduced for one daily session. To maintain performance on the task, animals received baseline training on all days in which rats were not at the proestrous stage. Therefore, rats received 3 days of baseline training between each behavioral challenge.

*Baseline.* Light stimulus lasted for 0.5 sec. A 5 sec ITI was presented before onset of stimulus. Animal was given 5 sec to respond before an omission was counted. Each session consisted of 100 trials. Rats were tested under baseline conditions until proestrus occurred. Data from this daily session was used for analysis. During each following proestrus, rats were tested under behavioral challenge conditions in the following order:

*Short stimulus.* Light stimulus duration was shortened from 0.5 sec to 0.25 sec. All other parameters were the same as baseline. This condition challenges attentional performance because of the decrease in the duration of the stimuli.

*Unpredictable short ITI.* Time before the onset of the light stimulus was pseudorandomly shortened to 1.5, 2.0, 3.0, or 4.5 sec distributed across the 100 trials. All other parameters were the same as baseline. This condition challenges attentional

performance because of the decrease in time between trials and the decrease in predictability of the stimuli.

*Unpredictable long ITI.* Time before the onset of the light stimulus was pseudorandomly lengthened to 4.5, 5.5, 6.5, or 7.5 sec distributed across the 100 trials. All other parameters were the same as baseline. This condition challenges attentional performance and impulsive action control because of the increase in time between trials and the decrease in predictability of the stimuli.

*Distracting noise.* Bursts of white noise (0.5 sec, 85 dB, 800 Hz) were presented at various time points during the 5 sec ITI (0.5, 2.5, 3.5, or 4.5 sec after start of the ITI). In 20% of the trials, no noise bursts were presented. All other parameters were the same as baseline. This condition challenges attentional performance because animals have to selectively ignore the distracting noise while still attending to the stimuli.

#### *Behavioral measures*

Throughout testing, the following behavioral measures were recorded by automated computer software (ABET II, Lafayette Instruments) on a PC connected to the conditioning chambers.

*Premature responses.* This cumulative measure is the total number of trials in which a rat nose poked into an aperture during the ITI. This reflects deficits in inhibitory control processes of response preparation and is the primary measure of impulsive action in this task.

*Perseverative responses.* This cumulative measure is the total number of additional nose pokes made into the apertures following either a correct or an incorrect

response. This reflects inhibitory control processes of response control and is the secondary measure of impulsive action in this task.

*Percent correct.* This cumulative measure is the total number of correct responses relative to the total number of trials completed. It indicates overall attentional performance during the task where attention must be sustained and divided across several spatial locations and is the primary measure of attentional performance on this task.

*Percent omissions.* This cumulative measure is the percentage of trials in which a rat failed to respond during the limited hold period. This can reflect a failure to detect the stimulus due to inattentiveness or due to motivational and/or motor deficits. This distinction can be interpreted more conclusively by examining the speed measures. It is the secondary measure of attentional performance on this task.

*Speed.* Two measures of speed were collected. First, the time between the onset of the stimulus and a correct nose poke was measured as correct response latency. Second, the time between a correct nose poke and the retrieval of the food from the magazine was measured as reward latency. Differences in response latency can indicate changes in decisional mechanisms, whereas differences in reward latency can indicate changes in motivational factors. If both measures are affected, motivational and/or motor functions could be affected (Muir et al. 1996; Robbins 2002).

#### *Statistical analyses*

To assess the stability of baseline performance at the end of training, performances across the last five days of training were analyzed for all dependent

variables using overall repeated measures ANOVAs with day as the within-subjects factor and sex as the between-subjects factor. To assess any possible changes in baseline performance during behavioral challenge testing, performances across the initial baseline data and baseline data collected the day before each behavioral challenge condition were analyzed for all dependent variables using overall repeated measures ANOVAs with day as the within-subjects factor and sex as the between-subjects factor. To assess performance during behavioral challenges, performances across baseline data collected on the day of proestrus prior to the first behavioral challenge and data from all behavioral challenge conditions (Short Stimulus, Unpredictable Short ITI, Unpredictable Long ITI, Distracting Noise) were analyzed for all dependent variables using overall repeated measures ANOVAs with behavioral condition as the within-subjects factor and sex as the between-subjects factor. When a significant sex by behavioral condition interaction was discovered, t-tests were conducted comparing performances of males and females at each level of behavioral condition. When a main effect of sex was discovered during the Unpredictable Short or Long ITI conditions, performance was sorted by ITI duration in order to assess the separate effects of changes in event predictability and changes in event rate. Performance was analyzed using overall repeated measures ANOVAs with ITI duration as the within-subjects factor and sex as the between-subjects factor.

## Results

### *Performance on the 5-choice serial reaction time task*

Four male and five female animals failed to reach criterion level performance during training and were excluded from all analyses, resulting in the following final group numbers: male ( $n = 10$ ), female ( $n = 9$ ).

### ***Baseline training***

Animals successfully acquired the task, as indicated by criterion level performance, within 70 training sessions. Performance remained stable across the last five days of baseline training, and there were no significant sex differences on any measure during the last five days of training. In addition, no significant change in baseline performance occurred across baseline sessions conducted between behavioral challenge testing days. There was no main effect of sex on the two measures of speed, correct response latency and reward collection latency, indicating that there was no sex difference in motor function, sensory function, motivational factors, or the overall ability of the animals to perform the task (Robbins 2002).

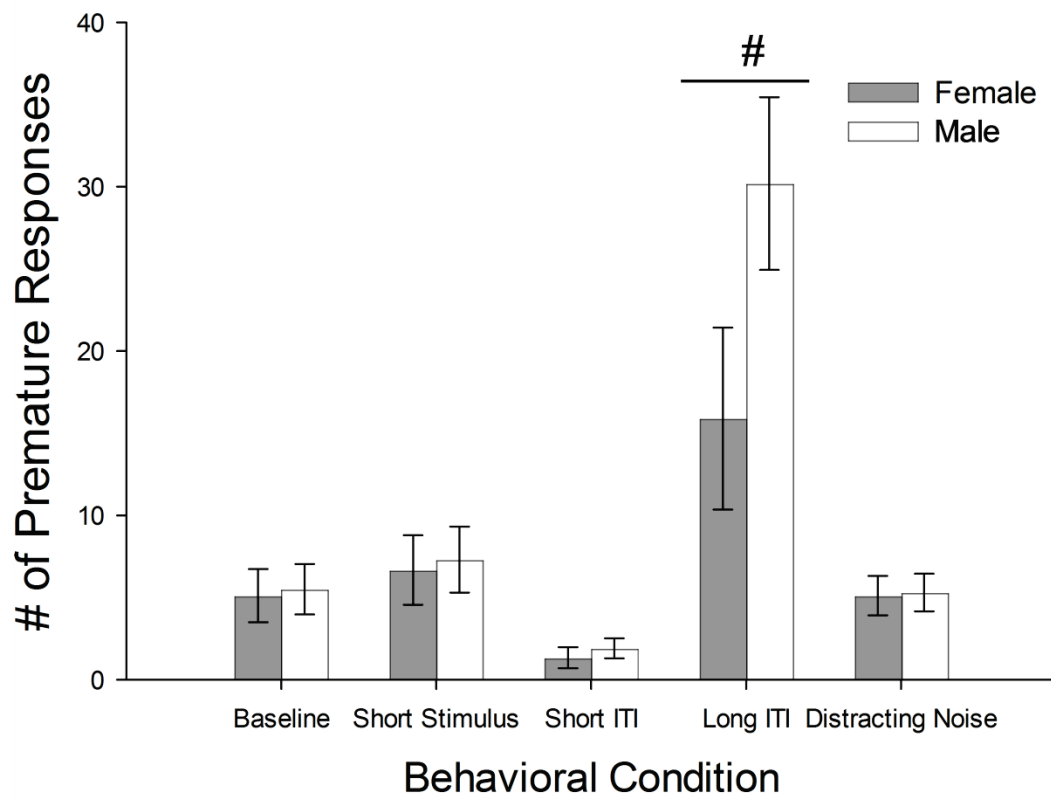
### ***Behavioral testing***

#### *Premature responses*

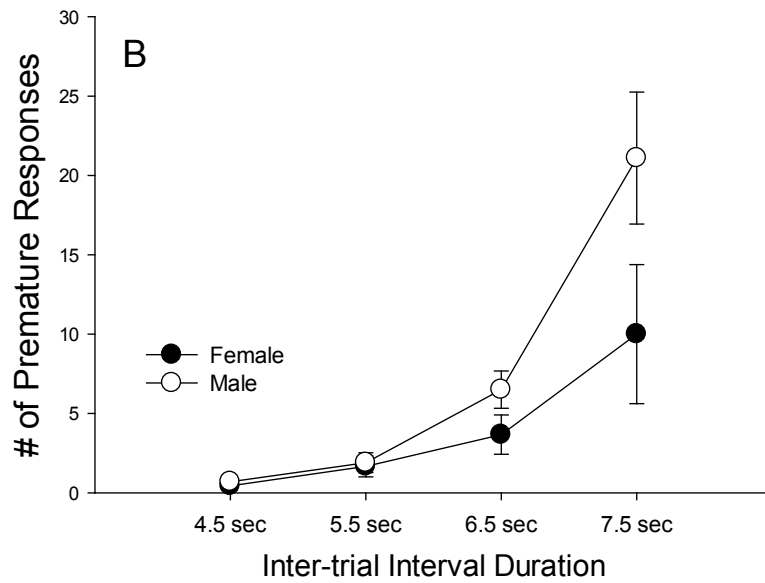
As illustrated in Figure 7, on the primary measure of impulsive action, premature responses, there was a significant main effect of behavioral condition ( $F(4,68) = 21.11$ ,  $p < 0.001$ ), indicating that impulsive action control was disrupted by the behavioral

challenges. There was a significant interaction between sex and behavioral condition ( $F(4,68) = 2.88, p = 0.029$ ), indicating that male impulsive action control was more disrupted by the behavioral challenges than was female impulsive action control. There was no significant main effect of sex across all behavioral conditions. As follow up to the significant interaction between sex and behavioral condition, t-tests revealed a trend to significance under the Unpredictable Long ITI ( $t(17) = 1.87, p = 0.078$ ), indicating that males made more premature responses than did females when the onset of the stimulus was unpredictably lengthened. No trends or sex differences were observed under other conditions. As illustrated in Figure 8, when performance during the Unpredictable Long ITI condition was analyzed across ITI durations, there was a significant main effect of ITI duration ( $F(3,51) = 21.55, p < 0.001$ ), indicating that impulsive action control was more disrupted during longer ITI durations. There was no main effect of sex across ITI durations, but there was a significant interaction between sex and ITI duration ( $F(3,51) = 3.08, p = 0.036$ ), indicating that longer ITI durations affected male impulsive action control more than female impulsive action control.

Figure 7



**Figure 7.** Number of premature responses across behavioral condition in adult male and female rats. Overall sex by behavioral condition interaction:  $p = 0.029$ . #  $p = 0.078$ , female performance compared to male performance during the Long ITI behavioral condition. (Bayless et al. 2012)

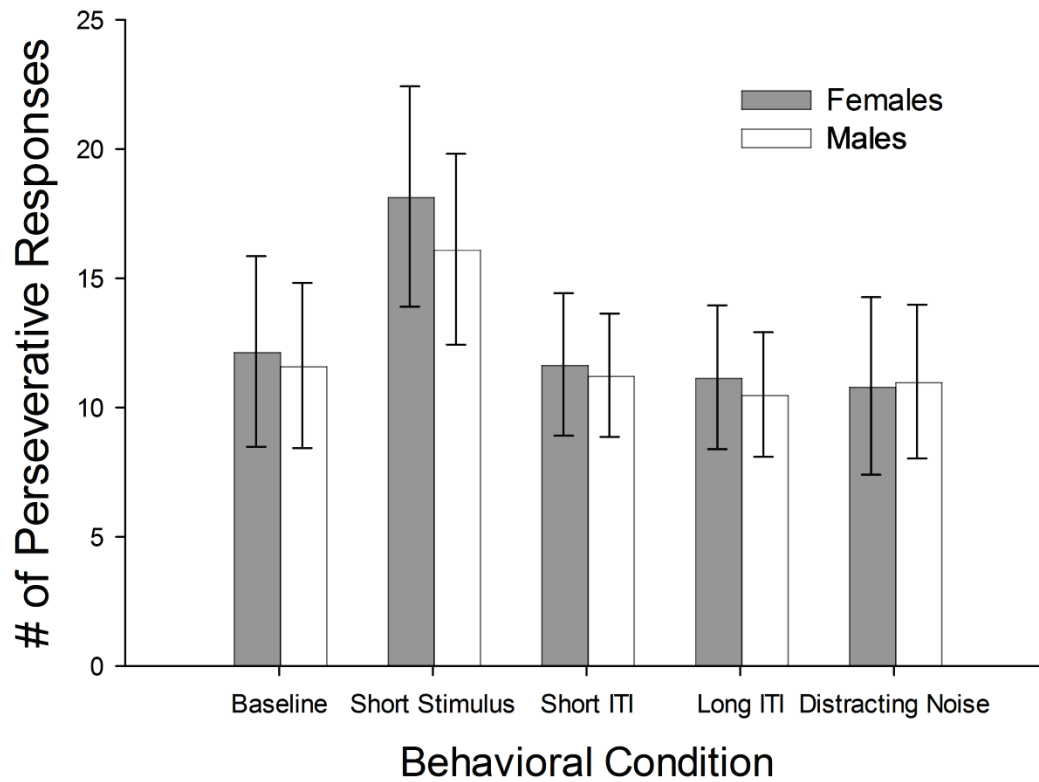
**Figure 8**

**Figure 8.** Number of premature responses across ITI durations in the Unpredictable Long ITI condition in adult male and female rats. Number of premature responses across ITI duration. Overall sex by behavioral condition interaction:  $p = 0.036$ . (Bayless et al. 2012)

### *Perseverative responses*

As illustrated in Figure 9, on the secondary measure of impulsive action, perseverative responses, there was no significant main effect of behavioral condition, sex, or interaction between sex and behavioral condition.

**Figure 9**

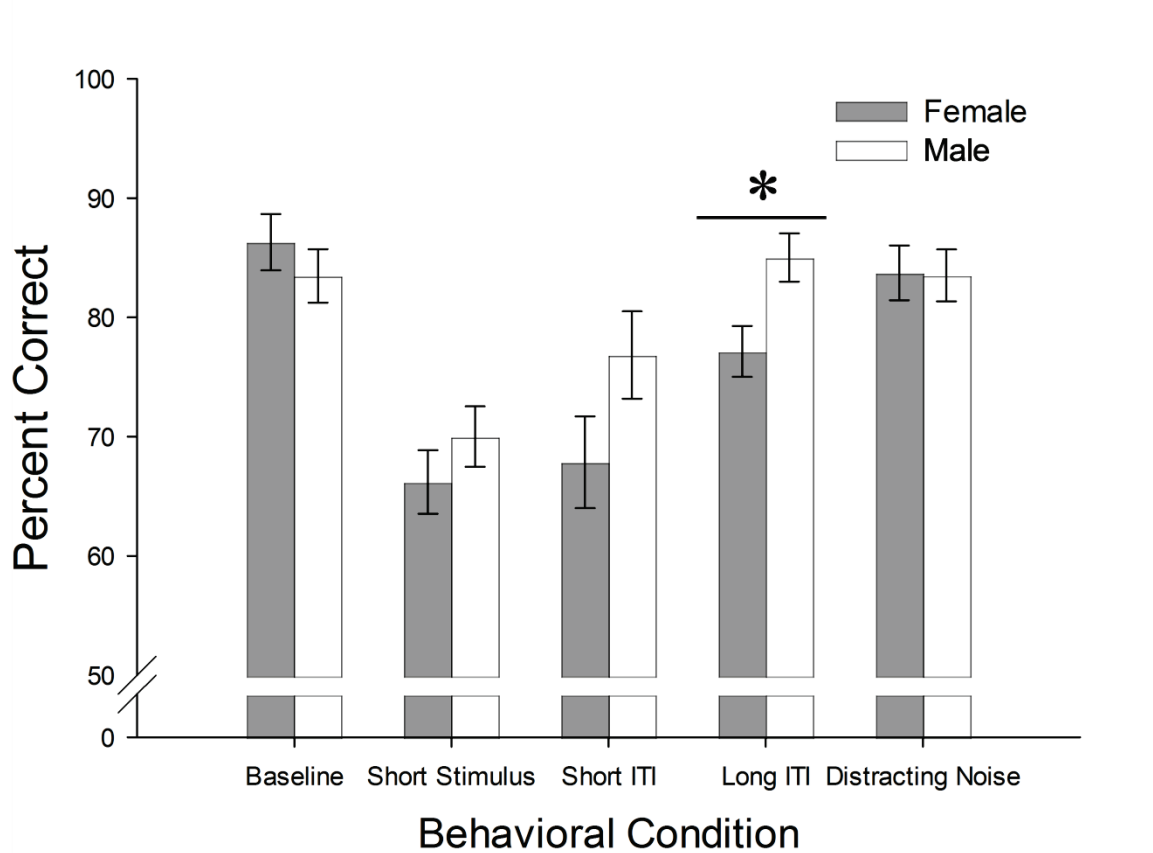


**Figure 9.** Number of perseverative responses across behavioral condition in adult male and female rats. (Bayless et al. 2012)

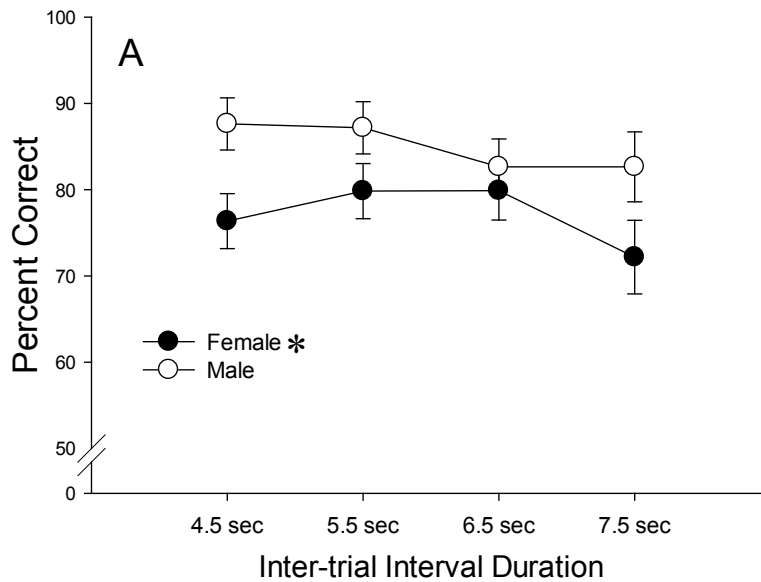
### *Percent correct*

As illustrated in Figure 10, on the primary measure of attentional performance, percent correct, there was a significant main effect of behavioral condition ( $F(4,68) = 22.31, p < 0.001$ ), indicating that attentional performance was disrupted by the behavioral challenges. There was also a significant interaction between sex and behavioral condition ( $F(4,68) = 2.64, p = 0.041$ ), indicating that female attentional performance was more disrupted by the behavioral challenges than was male attentional performance. There was no significant main effect of sex across all behavioral conditions. As follow up to the significant interaction between sex and behavioral condition, t-tests revealed that under the Unpredictable Long ITI condition males performed with a higher percentage of correct responses than did females ( $t(17) = 2.68, p = 0.016$ ). No sex differences were observed under other conditions. As illustrated in Figure 11, when performance during the Unpredictable Long ITI condition was analyzed across ITI durations, there was a significant main effect of sex ( $F(1,17) = 7.20, p = 0.016$ ) but no significant main effect of ITI duration or interaction between sex and ITI duration, indicating that the difference in attentional performance between males and females was due to changes in the predictability of the stimuli and not due changes in the ITI duration.

Figure 10



**Figure 10.** Percent correct across behavioral condition in adult male and female adult rats. Overall sex by behavioral condition interaction:  $p = 0.041$ . \*  $p < 0.05$ , female performance compared to male performance during the Long ITI behavioral condition. (Bayless et al. 2012)

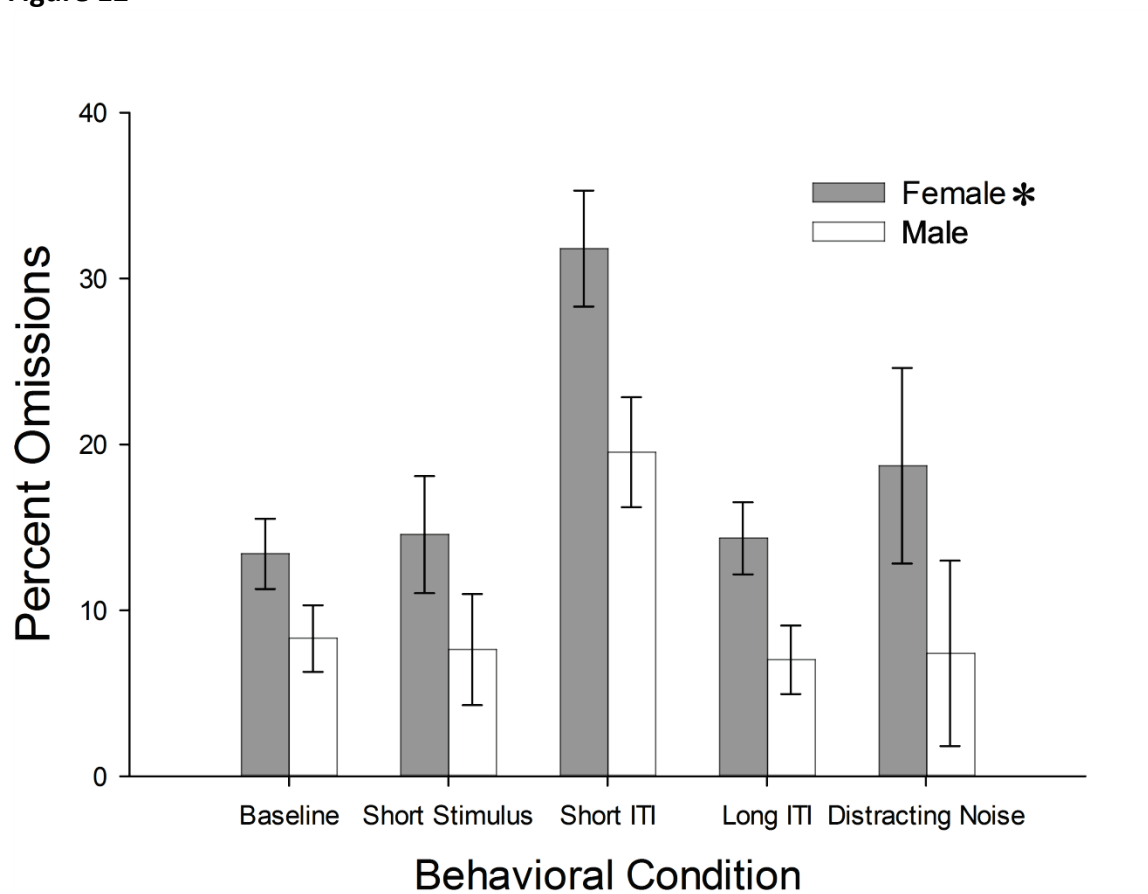
**Figure 11**

**Figure 11.** Percent correct across ITI durations in the Unpredictable Long ITI condition in adult male and female rats. Overall main effect of sex: \*  $p = 0.016$ . (Bayless et al. 2012)

### *Percent omissions*

As illustrated in Figure 12, on the secondary measure of attentional performance, percent omissions, there was a significant main effect of behavioral condition ( $F(4,68) = 8.84$ ,  $p < 0.001$ ), indicating that attentional performance was disrupted by the behavioral challenges. There was a significant main effect of sex ( $F(1,17) = 6.68$ ,  $p = 0.019$ ), indicating that females omitted a higher percentage of trials than did males across all behavioral conditions. There was no significant interaction between sex and behavioral condition.

Figure 12



**Figure 12.** Percent omissions across behavioral condition in adult male and female rats. Overall main effect of sex: \*  $p = 0.019$ . (Bayless et al. 2012)

### *Speed measures*

As shown in Table 1, on the two measures of speed, correct response latency and reward collection latency, there were no significant main effects of behavioral condition, sex, or interactions between sex and behavioral condition, indicating that there were no sex differences in motor function, sensory function, motivational factors, or the overall ability of the animals to perform the task during the behavioral challenges (Robbins 2002).

**Table 1**

**Table 1**

Reward collection latency and correct response latency across behavioural challenge condition

		<b>Baseline</b>		<b>Short Stimulus</b>		<b>Short ITI</b>		<b>Long ITI</b>		<b>Distracting Noise</b>	
		M	S.E.M.	M	S.E.M.	M	S.E.M.	M	S.E.M.	M	S.E.M.
<b>Correct latency (sec)</b>											
	<b>Female</b>	0.69	0.04	0.68	0.05	0.77	0.04	0.68	0.04	0.67	0.04
	<b>Male</b>	0.65	0.03	0.66	0.04	0.79	0.04	0.64	0.03	0.65	0.04
<b>Reward latency (sec)</b>											
	<b>Female</b>	1.01	0.04	0.97	0.04	0.94	0.04	1.02	0.04	1.01	0.05
	<b>Male</b>	1.01	0.03	0.99	0.03	0.98	0.03	1.03	0.03	0.98	0.04

(Bayless et al. 2012)

**Aim 3: To identify possible sex differences in dopaminergic and noradrenergic reuptake in the orbital frontal cortex and dorsal striatum**

**Experiment 3: Sex differences in markers of dopaminergic and noradrenergic reuptake in the OFC and dSTR**

**Experiment 3a: Sex differences in DAT and NET in prepubertal rats**

*Hypothesis*

High levels of norepinephrine in the OFC are associated with increased inhibitory control. Mixed results are reported in terms of the direction of effects for dopamine. I hypothesized that control female rats would have increased levels of dopamine transporter (DAT) and norepinephrine transporter (NET) in the OFC and the dSTR as compared to control male rats and female rats treated with neonatal testosterone, indicating greater dopaminergic and noradrenergic neurotransmission, which could contribute to increased inhibitory control.

**Methods**

*Tissue dissection and processing*

One day following the completion of behavior testing in the preliminary studies at pnd 35, male and female control rats and female rats treated with neonatal testosterone animals were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) and killed by decapitation. Whole brains were

removed, quick-frozen on dry ice, and stored at -80°C until use in the present experiment. In a cryostat at -20°C, the OFC and dSTR were dissected (As described in Spijker 2011) and stored at -80°C until processing. Tissue was homogenized in 20 µl/mg lysis buffer containing 1mM EGTA, 1mM EDTA, 20 mM Tris, 1 mM sodium pyrophosphate tetrabasic decahydrate, 4 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 0.1 µM microcystin, and 1% protease inhibitor cocktail (Sigma-Aldrich). Samples were then centrifuged for 15 min at 1000 x g at 4 C, protein concentration of supernatants was determined (Bradford Protein Assay Kit; Pierce, Rockford, IL), and each sample was diluted 1:1 with Laemmli Sample Buffer (Bio-Rad; Hercules, CA), mixed with 350 mM D,L-dithiothreitol, boiled for 5 min, and stored at -80 C.

#### *Proteins of interest*

Western blotting procedures were used to assess sex differences and the influence of neonatal testosterone exposure on protein levels of DAT and NET in the OFC and dSTR of prepubertal rats.

#### *Electrophoresis and immunostaining*

For each DAT and NET sample, 25 µg of total protein was loaded and separated at 200 V on 10% SDS-PAGE gels (Bio-Rad) for 60 min. Molecular weight markers (Kaleidoscope; Bio-Rad) were included with each run. Proteins were transferred to nitrocellulose membranes at 100V for 60 min. Membranes were then blocked with 5% nonfat dry milk in 0.1% Tween/1 X Tris-buffered saline (TTBS) at room temperature for 60 min. Following this, membranes were cut to separate the section containing the proteins of interest from the section containing the loading control β-actin. Membranes

were then incubated with primary antibodies for DAT (rabbit polyclonal, 1:2000; Millipore, AB2231), NET (rabbit polyclonal, 1:2000; Millipore, AB2234), or  $\beta$ -actin (mouse monoclonal; 1:15,000; Santa Cruz) overnight at 4 C in 1% nonfat dry milk-TTBS. Blots were washed three times for 15 min each with TTBS and incubated with 5% nonfat dry milk containing goat anti-rabbit IgG (DAT, 1:10,000; NET, 1:10,000; Santa Cruz) or goat anti-mouse IgG ( $\beta$ -actin, 1:10,000; Santa Cruz) conjugated to horseradish peroxidase for 1.5 h at room temperature. Blots were washed again three times for 15 min each and incubated for 1 min with the chemiluminescent substrate Pierce ECL western blotting substrate (DAT; NET;  $\beta$ -actin; Fisher Scientific) and exposed to film (Kodak Biomax MR) for varying durations to capture optimal signal intensity. Films were imaged using MCID Core imaging software (InterFocus Imaging Ltd., Cambridge, England), and optical density x area was measured for bands of interest. All values were represented as a percentage relative to  $\beta$ -actin for each sample.

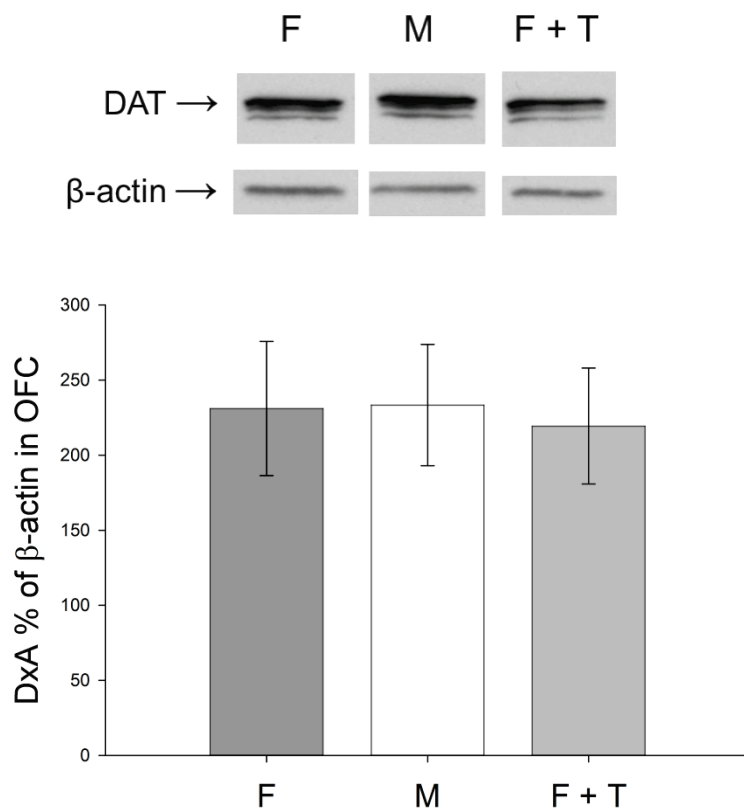
#### *Statistical analyses*

The optical density x area values as a percentage of  $\beta$ -actin were analyzed using a one-way ANOVA for each protein of interest. When a significant main effect of treatment was discovered, Fisher's LSD pair-wise comparison procedures were conducted.

## Results

### *DAT in OFC in prepubertal rats*

Western blots revealed a band of DAT-like immunoreactivity at approximately 80 kDa. As illustrated in Figure 13, analysis of protein levels of DAT in the OFC of control male, control female, and testosterone-treated female prepubertal rats revealed no main effect of treatment. There was no effect of neonatal treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect DAT protein expression in the OFC of prepubertal rats.

**Figure 13**

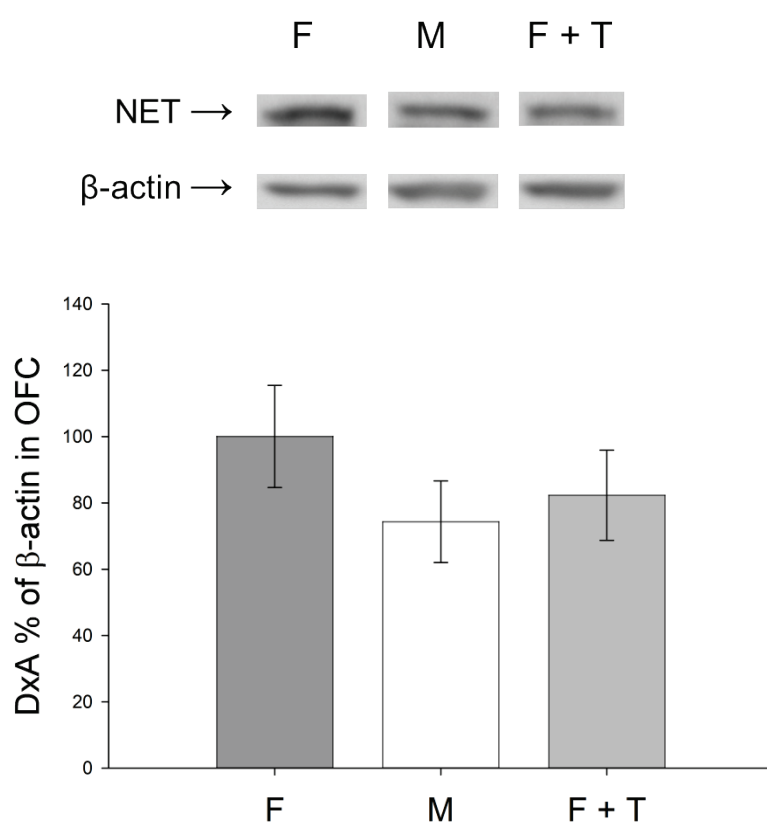
**Figure 13.** Western blot data showing the effect of neonatal hormone environment on protein levels of dopamine transporter (DAT) in the orbital frontal cortex (OFC) of prepubertal rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for DAT and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

#### *NET in OFC in prepubertal rats*

Western blots revealed a band of NET-like immunoreactivity at approximately 69 kDa. As illustrated in Figure 14, analysis of protein levels of NET in the OFC of control male, control female, and testosterone-treated female prepubertal rats revealed no

main effect of treatment. There was no effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect NET protein expression in the OFC of prepubertal rats.

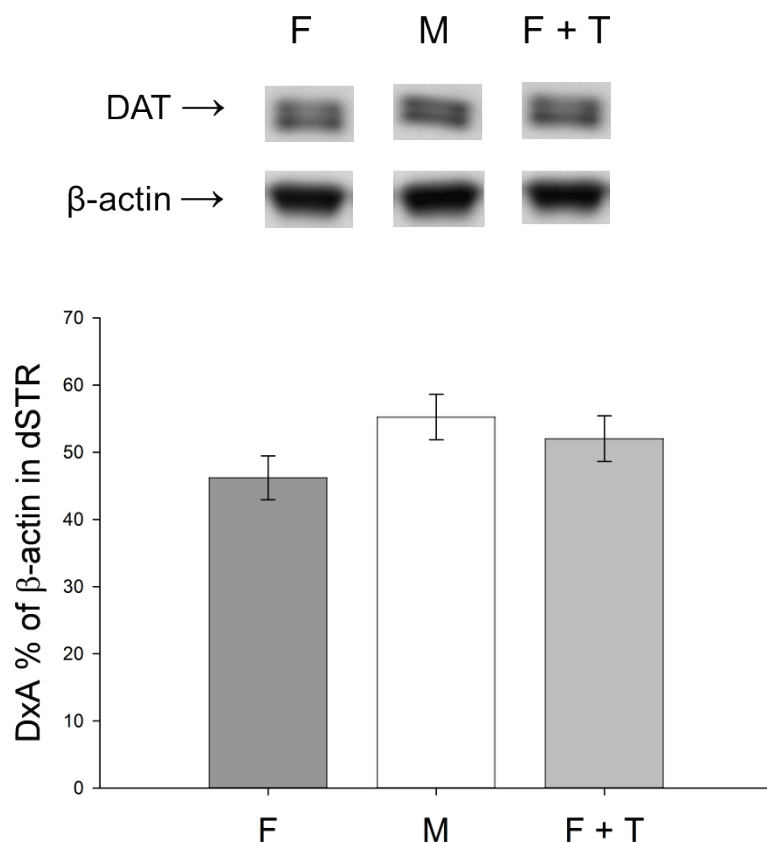
**Figure 14**



**Figure 14.** Western blot data showing the effect of neonatal hormone environment on protein levels of norepinephrine transporter (NET) in the orbital frontal cortex (OFC) of prepubertal rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for NET and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

*DAT in dSTR in prepubertal rats*

Western blots revealed a band of DAT-like immunoreactivity at approximately 80 kDa. As illustrated in Figure 15, analysis of protein levels of DAT in the dSTR of control male, control female, and testosterone-treated female prepubertal rats revealed no main effect of treatment. There was no effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect DAT protein expression in the dSTR of prepubertal rats.

**Figure 15**

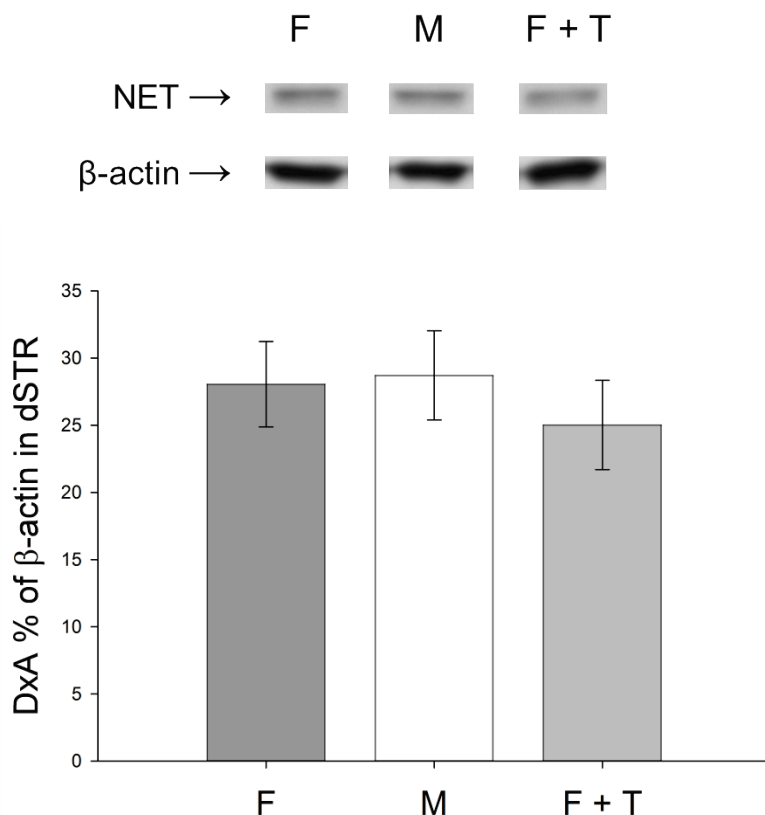
**Figure 15.** Western blot data showing the effect of neonatal hormone environment on protein levels of dopamine transporter (DAT) in the dorsal striatum (dSTR) of prepubertal rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for DAT and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

#### *NET in dSTR in prepubertal rats*

Western blots revealed a band of NET-like immunoreactivity at approximately 69 kDa. As illustrated in Figure 16, analysis of protein levels of NET in the dSTR of control

male, control female, and testosterone-treated female prepubertal rats revealed no main effect of treatment. There was no effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect NET protein expression in the dSTR of prepubertal rats.

**Figure 16**



**Figure 16.** Western blot data showing the effect of neonatal hormone environment on protein levels of norepinephrine transporter (NET) in the dorsal striatum (dSTR) of prepubertal rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for NET and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

### **Experiment 3b: Sex differences in DAT and NET in adult rats**

#### *Hypothesis*

I hypothesized that adult female rats would have increased levels of DAT and NET in the OFC and the dSTR as compared to adult male rats, indicating greater dopaminergic and noradrenergic neurotransmission leading to increased inhibitory control.

#### **Methods**

##### *Tissue dissection and processing*

One day following the completion of behavior testing in the preliminary studies at pnd 85, adult male and female rats were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) and killed by decapitation. Whole brains were removed, quick-frozen on dry ice, and stored at -80°C until use in the present experiment. Tissue dissection and processing was the same as described in Experiment 3a.

##### *Proteins of interest*

Western blotting procedures were used to assess sex differences in protein levels of DAT and NET in the OFC and dSTR of adult rats.

##### *Electrophoresis and immunostaining*

Western blotting and imaging procedures were the same as described in Experiment 3a.

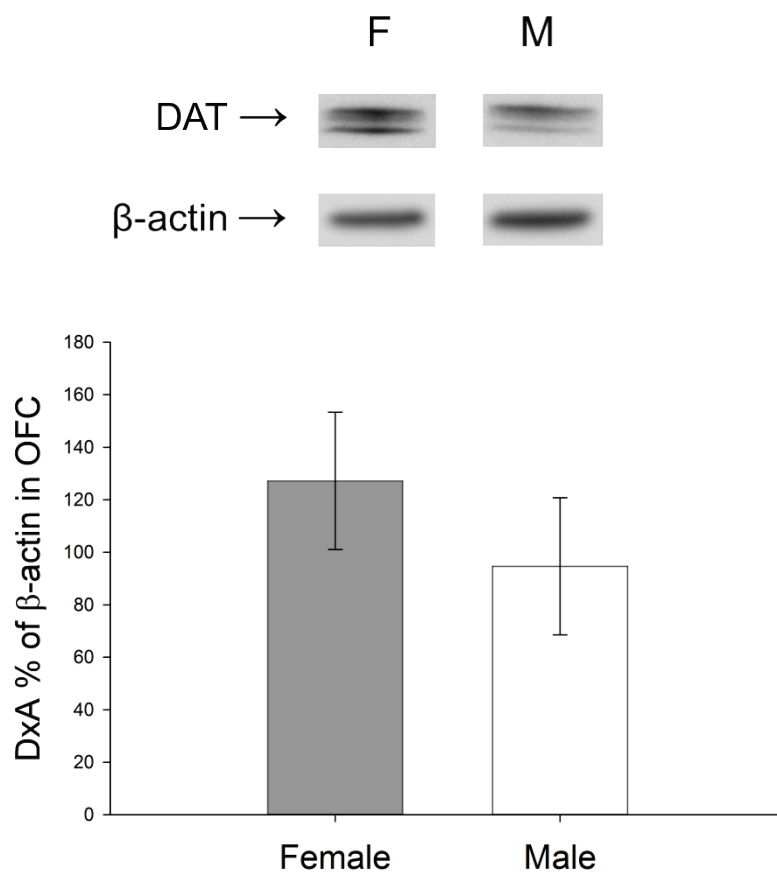
### *Statistical analyses*

The optical density x area values as a percentage of  $\beta$ -actin were analyzed using a one-way ANOVA for each protein of interest.

## **Results**

### *DAT in OFC in adult rats*

Western blots revealed a band of DAT-like immunoreactivity at approximately 80 kDa. As illustrated in Figure 17, analysis of protein levels of DAT in the OFC of male and female adult rats revealed no main effect of sex. There was no effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex does not affect DAT protein expression in the OFC of adult rats.

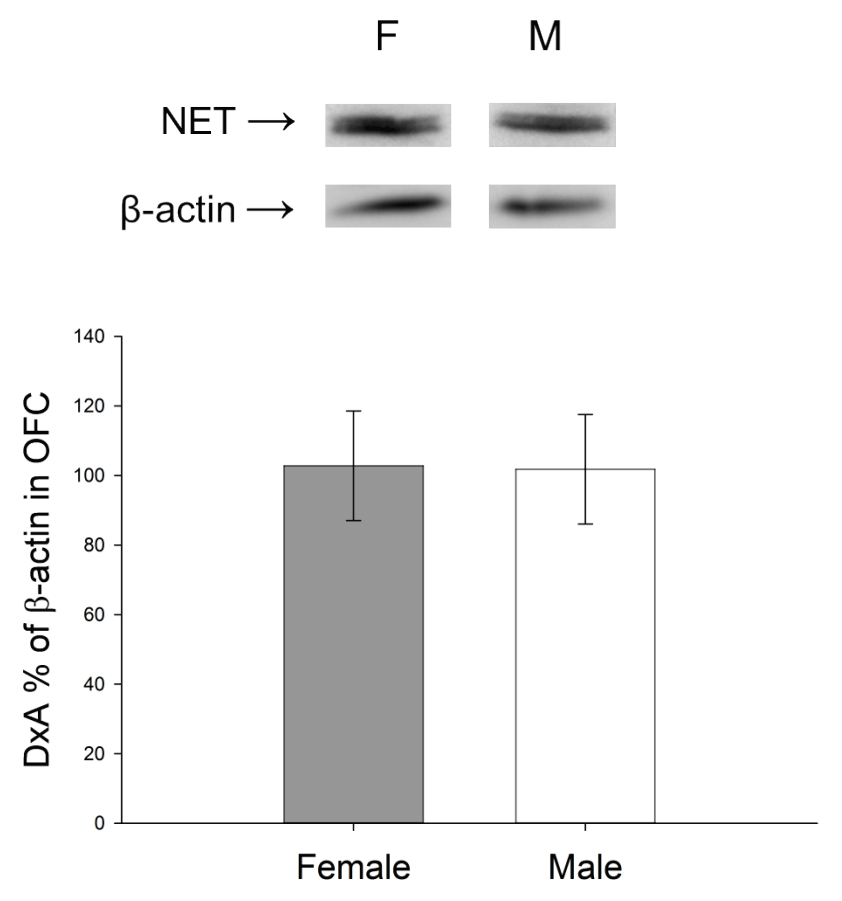
**Figure 17**

**Figure 17.** Western blot data showing the effect of sex on protein levels of dopamine transporter (DAT) in the orbital frontal cortex (OFC) of adult rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for DAT and the loading control  $\beta$ -actin are shown in insets above the graph.

### NET in OFC in adult rats

Western blots revealed a band of DAT-like immunoreactivity at approximately 69 kDa. As illustrated in Figure 18, analysis of protein levels of NET in the OFC of male and female adult rats revealed no main effect of sex. There was no effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex does not affect NET protein expression in the OFC of adult rats.

**Figure 18**



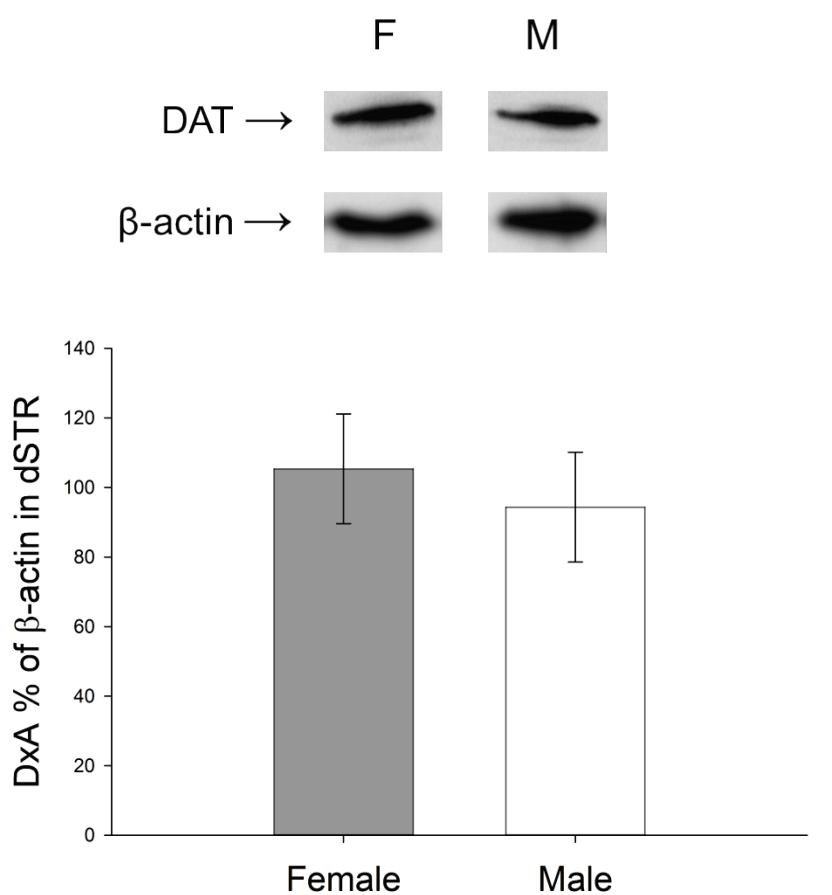
**Figure 18.** Western blot data showing the effect of sex on protein levels of norepinephrine transporter (NET) in the orbital frontal cortex (OFC) of adult rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for NET and the loading control  $\beta$ -actin are shown in insets above the graph.

### *DAT in dSTR in adult rats*

Western blots revealed a band of DAT-like immunoreactivity at approximately 80 kDa. As illustrated in Figure 19, analysis of protein levels of DAT in the dSTR of male and female adult rats revealed no main effect of sex. There was no effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals.

Results indicate that sex does not affect DAT protein expression in the dSTR of adult rats.

**Figure 19**



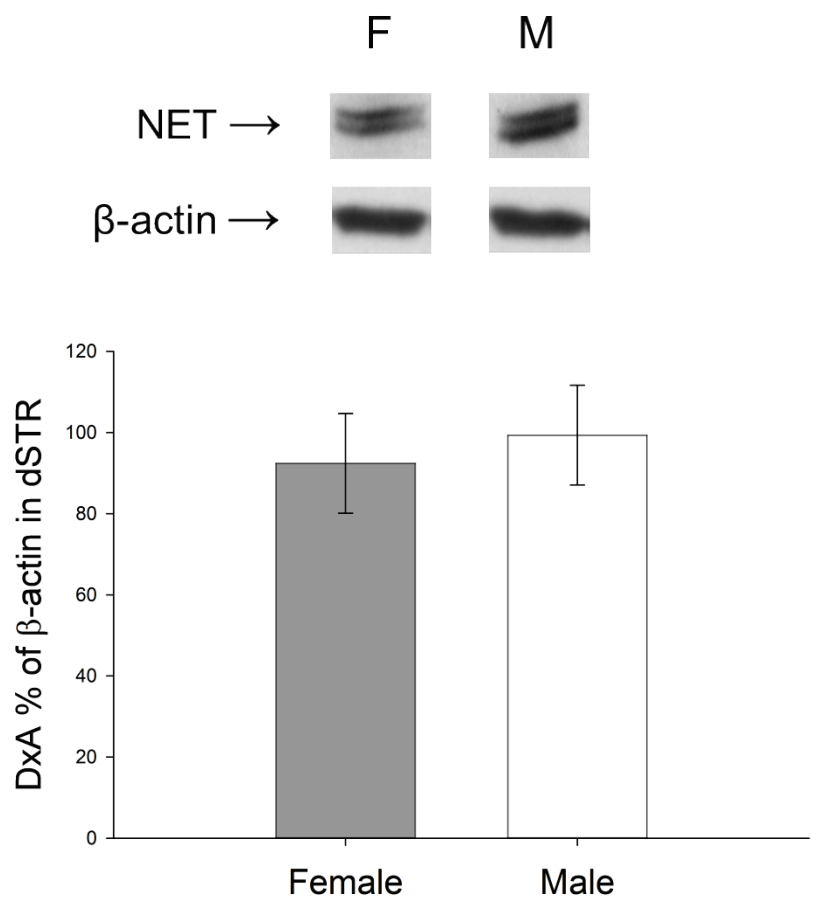
**Figure 19.** Western blot data showing the effect of sex on protein levels of dopamine transporter (DAT) in the dorsal striatum (dSTR) of adult rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for DAT and the loading control  $\beta$ -actin are shown in insets above the graph.

### NET in dSTR in adult rats

Western blots revealed a band of DAT-like immunoreactivity at approximately 69 kDa. As illustrated in Figure 20, analysis of protein levels of NET in the dSTR of male and female adult rats revealed no main effect of sex. There was no effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals.

Results indicate that sex does not affect NET protein expression in the dSTR of adult rats.

**Figure 20**



**Figure 20.** Western blot data showing the effect of sex on protein levels of norepinephrine transporter (NET) in the dorsal striatum (dSTR) of adult rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for NET and the loading control  $\beta$ -actin are shown in insets above the graph.

**Aim 4: To examine if greater orbital frontal cortex control over the dorsal striatum in females as compared to males provides females with a greater ability than males to inhibit undesirable behaviors**

**Experiment 4a: Sex differences in markers of myelination in the OFC and dSTR**

**Experiment 4a*i*: Sex differences in MBP and PLP in prepubertal rats**

*Hypothesis*

Increased levels of myelin basic protein (MBP) and myelin proteolipid protein (PLP) are indicative of increased myelin levels. Increased myelin levels indicate either an increase in neuronal connections or an increase in the axonal diameter of neurons. Both the number of neuronal connections and axonal diameter are associated with faster, more efficient neuronal communication. I hypothesized that prepubertal female rats would have increased levels of MBP and PLP in the OFC but not the dSTR as compared to males, indicating that neurotransmission is faster in the OFC but similar in the dSTR in females as compared to males perhaps leading to increased OFC communication with and control over the dSTR in females as compared to males.

**Methods**

*Tissue dissection and processing*

The same brain tissue from Experiments 3a was used.

### *Proteins of interest*

Western blotting procedures were used to assess sex differences in protein levels of MBP and PLP in the OFC and dSTR of prepubertal rats.

### *Electrophoresis and immunostaining*

For each MBP and PLP sample, 25 µg of total protein was loaded and separated at 200 V on 15% SDS-PAGE gels (Bio-Rad) for 60 min. Molecular weight markers (Kaleidoscope; Bio-Rad) were included with each run. Proteins were transferred to nitrocellulose membranes at 100V for 60 min. Membranes were blocked with 5% nonfat dry milk in 0.1% Tween/1 X Tris-buffered saline (TTBS) at room temperature for 60 min. Following this, membranes were cut to separate the section containing the proteins of interest from the section containing the loading control  $\beta$ -actin. Membranes were then incubated with primary antibodies for MBP (mouse monoclonal; 1:5000, Abcam, AB78156), PLP (mouse monoclonal; 1:1500, Millipore, MAB388), or  $\beta$ -actin (mouse monoclonal; 1:15,000; Santa Cruz) overnight at 4 C in 1% nonfat dry milk-TTBS. Blots were washed three times for 15 min each with TTBS and incubated with 5% nonfat dry milk containing goat antimouse IgG (MBP, 1:10,000; PLP, 1:10,000;  $\beta$ -actin, 1:10,000; Santa Cruz) conjugated to horseradish peroxidase for 1.5 h at room temperature. Blots were washed again three times for 15 min each and incubated for 1 min with the chemiluminescent substrate Pierce ECL western blotting substrate (MBP;  $\beta$ -actin; Fisher Scientific) or 5 min with the chemiluminescent substrate SuperSignal West Femto (PLP; Fisher Scientific) and exposed to film (Kodak Biomax MR) for varying durations to capture optimal signal intensity. Films were imaged using MCID Core imaging software

(InterFocus Imaging Ltd., Cambridge, England), and optical density x area was measured for bands of interest. All values were represented as a percentage relative to  $\beta$ -actin for each sample.

### *Statistical analyses*

For PLP data, the optical density x area values as a percentage of  $\beta$ -actin were analyzed using a one-way ANOVA. For MBP data, the optical density x area values as a percentage of  $\beta$ -actin was analyzed using an overall repeated measures ANOVAs with isoform as the within-subjects factor and treatment as the between-subjects factor. When a significant main effect of treatment was discovered, Fisher's LSD pair-wise comparison procedures were conducted.

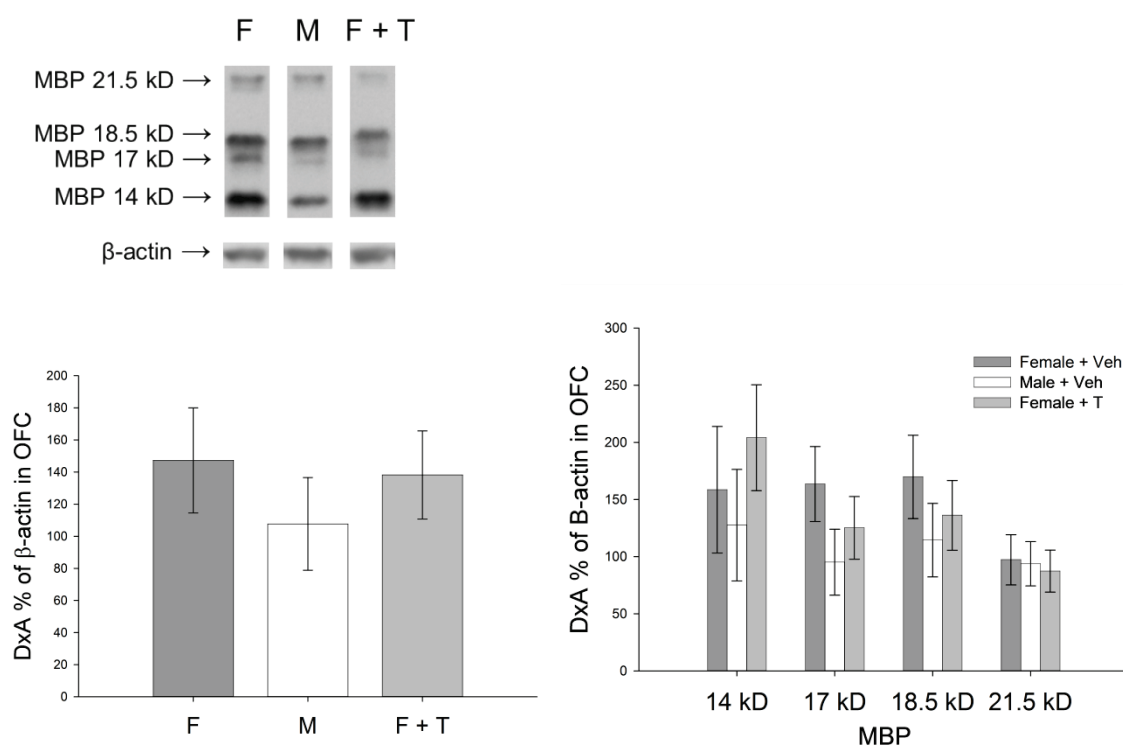
## **Results**

### *MBP in OFC in prepubertal rats*

Western blots revealed four isoform bands of MBP-like immunoreactivity at approximately 21.5, 18.5, 17.0, and 14.0 kDa. As illustrated in Figure 21, analysis of protein levels of MBP in the OFC of control male, control female, and testosterone-treated female prepubertal rats revealed a main effect of MBP isoform ( $F(3,69) = 5.69$ ,  $p = 0.002$ ), indicating that protein levels varied across the four isoforms. However, there was no main effect of treatment or interaction between treatment and MPB isoform. There was no main effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and

neonatal testosterone exposure do not affect MBP protein expression in the OFC of prepubertal rats.

**Figure 21**



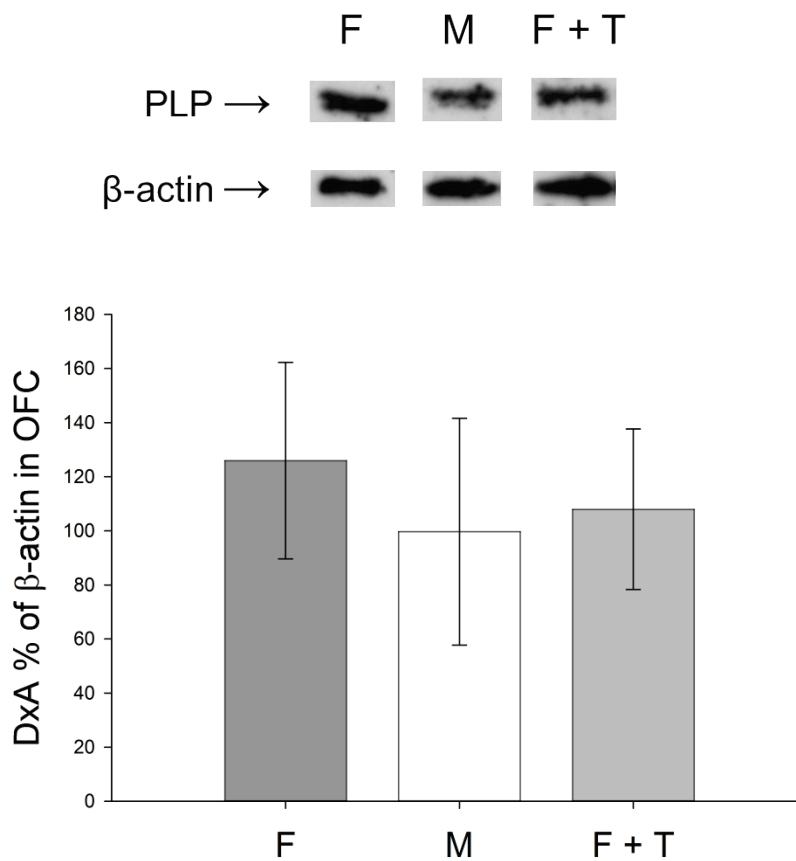
**Figure 21.** Western blot data showing the effect of neonatal hormone environment on protein levels of myelin basic protein (MBP) with isoforms averaged (left) and isolated (right) in the orbital frontal cortex (OFC) of prepubertal rats,. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for MBP and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

#### *PLP in OFC in prepubertal rats*

Western blots revealed a band of PLP-like immunoreactivity at approximately 23 kDa. As illustrated in Figure 22, analysis of protein levels of PLP in the OFC of control

male, control female, and testosterone-treated female prepubertal rats revealed no main effect of treatment. There was no main effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect PLP protein expression in the OFC of prepubertal rats.

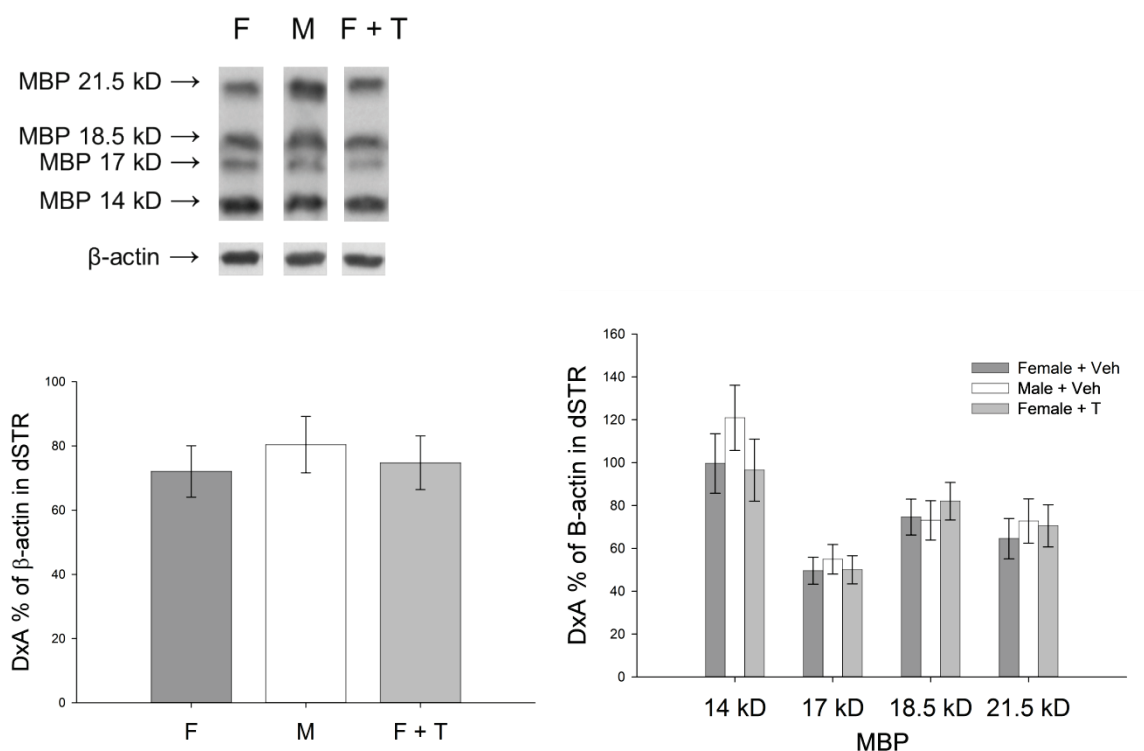
**Figure 22**



**Figure 22.** Western blot data showing the effect of neonatal hormone environment on protein levels of myelin proteolipid protein (PLP) in the orbital frontal cortex (OFC) of prepubertal rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for PLP and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

*MBP in dSTR in prepubertal rats*

Western blots revealed four isoform bands of MBP-like immunoreactivity at approximately 21.5, 18.5, 17.0, and 14.0 kDa. As illustrated in Figure 23, analysis of protein levels of MBP in the dSTR of control male, control female, and testosterone-treated female prepubertal rats revealed a main effect of MBP isoform ( $F(3,90) = 30.72$ ,  $p < 0.001$ ), indicating that protein levels varied across the four isoforms. However, there was no main effect of treatment or interaction between treatment and MPB isoform. There was no main effect of treatment on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect MBP protein expression in the dSTR of prepubertal rats.

**Figure 23**

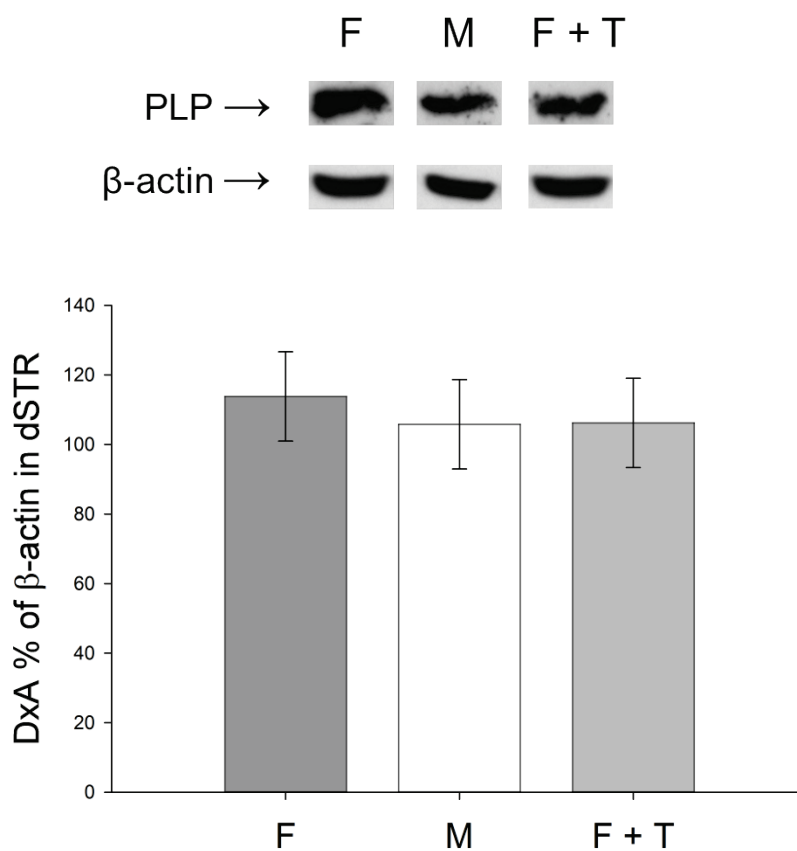
**Figure 23.** Western blot data showing the effect of neonatal hormone environment on protein levels of myelin basic protein (MBP) with isoforms averaged (left) and isolated (right) in the dorsal striatum (dSTR) of prepubertal rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for MBP and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

#### *PLP in dSTR in prepubertal rats*

Western blots revealed a band of PLP-like immunoreactivity at approximately 23 kDa. As illustrated in Figure 24, analysis of protein levels of PLP in the dSTR of control male, control female, and testosterone-treated female prepubertal rats revealed no main effect of treatment. There was no main effect of treatment on levels of  $\beta$ -actin,

indicating that equal amounts of total protein were loaded for all animals. Results indicate that sex and neonatal testosterone exposure do not affect PLP protein expression in the dSTR of prepubertal rats.

**Figure 24**



**Figure 24.** Western blot data showing the effect of neonatal hormone environment on protein levels of myelin proteolipid protein (PLP) in the dorsal striatum (dSTR) of prepubertal rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for PLP and the loading control  $\beta$ -actin are shown in insets above the graph. F: control female; M: control male; F + T: neonatal testosterone-treated female

### **Experiment 4a<sub>ii</sub>: Sex differences in MBP and PLP in adult rats**

#### *Hypothesis*

I hypothesized that adult female rats would have increased levels of MBP and PLP in the OFC but not the dSTR as compared to males, indicating that neurotransmission is faster in the OFC but similar in the dSTR in females as compared to males perhaps leading to increased OFC communication with and control over the dSTR in females as compared to males.

#### **Methods**

##### *Tissue dissection and processing*

The same brain tissue from Experiments 3b was used.

##### *Proteins of interest*

Western blotting procedures were used to assess sex differences in protein levels of MBP and PLP in the OFC and dSTR of adult rats.

##### *Electrophoresis and immunostaining*

Western blotting and imaging procedures were the same as described in Experiment 4a<sub>i</sub>.

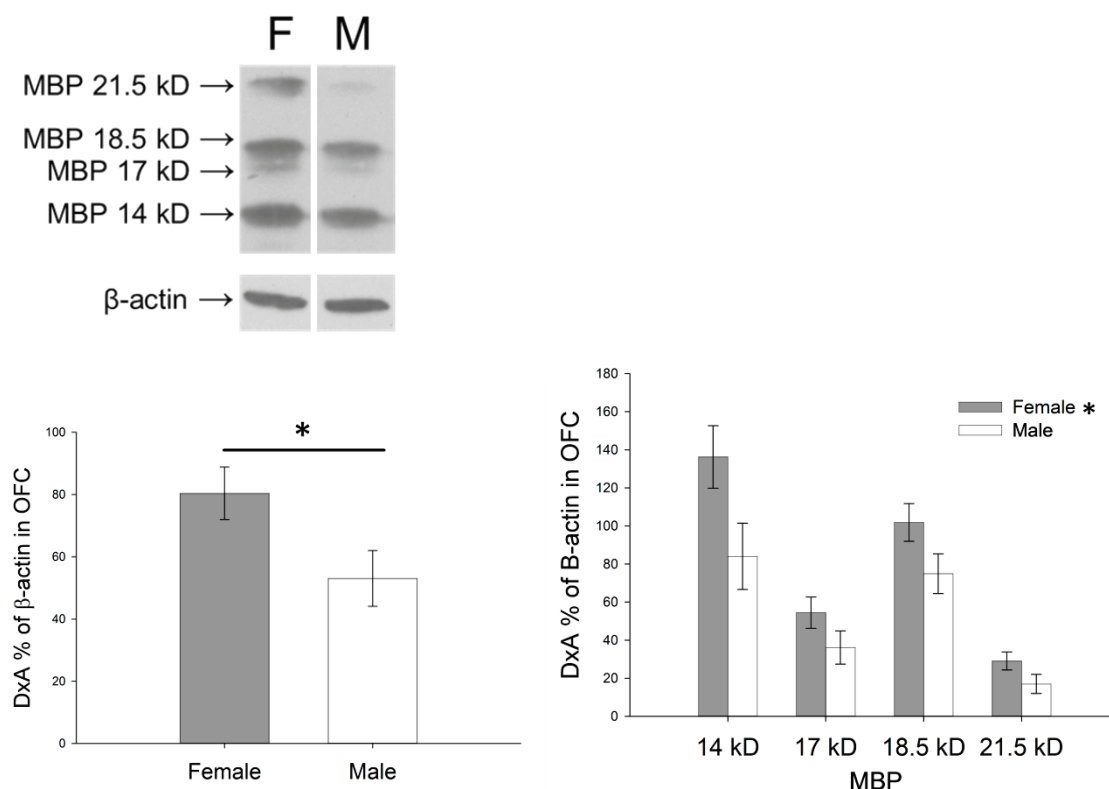
##### *Statistical analyses*

For PLP data, the optical density x area values as a percentage of  $\beta$ -actin were analyzed using a one-way ANOVA. For MBP data, the optical density x area values were analyzed using an overall repeated measures ANOVAs with isoform as the within-subjects factor and sex as the between-subjects factor.

## Results

### *MBP in OFC in adult rats*

Western blots revealed four isoform bands of MBP-like immunoreactivity at approximately 21.5, 18.5, 17.0, and 14.0 kDa. As illustrated in Figure 25, analysis of protein levels of MBP in the OFC of male and female adult rats revealed a main effect of MBP isoform ( $F(3,45) = 51.17$ ,  $p < 0.001$ ) and a main effect of sex ( $F(1,15) = 4.95$ ,  $p = 0.042$ ). There was no interaction between sex and MPB isoform. There was no main effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that expression of MBP in the OFC is significantly greater in adult female rats than in adult male rats.

**Figure 25**

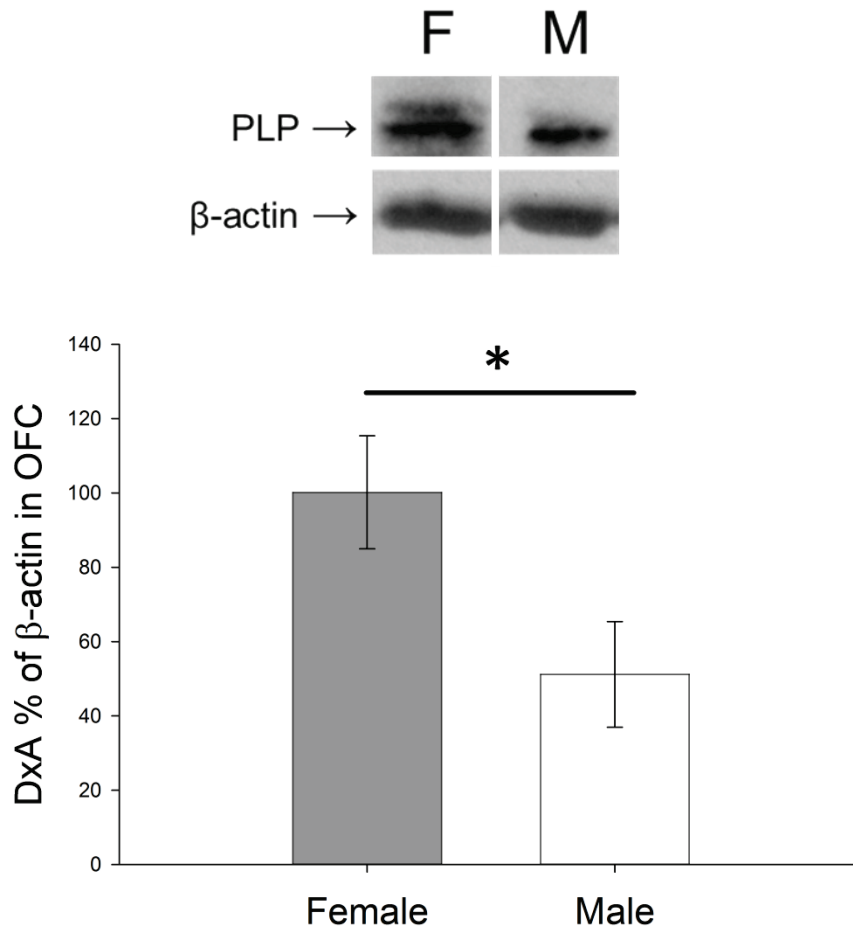
**Figure 25.** Western blot data showing the effect of sex on protein levels of myelin basic protein (MBP) with isoforms averaged (left) and isolated (right) in the orbital frontal cortex (OFC) of adult rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for MBP and the loading control  $\beta$ -actin are shown in insets above the graph. Overall main effect of sex: \*  $p < 0.05$ .

#### *PLP in OFC in adult rats*

Western blots revealed a band of PLP-like immunoreactivity at approximately 23 kDa. As illustrated in Figure 26, analysis of protein levels of PLP in the OFC of male and female adult rats revealed a main effect of sex ( $F(1,13) = 5.53$ ,  $p = 0.035$ ). There was no main effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein

were loaded for all animals. Results indicate that expression of PLP in the OFC is significantly greater in adult female rats than in adult male rats.

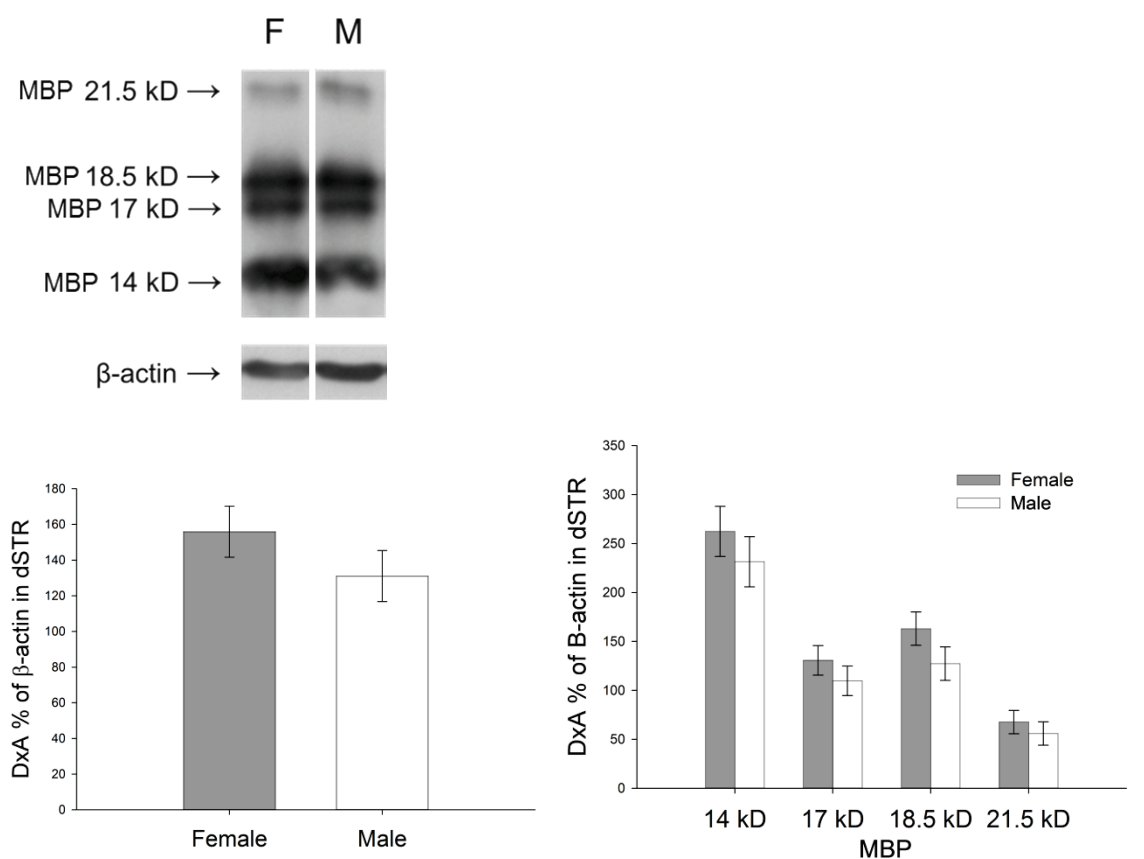
**Figure 26**



**Figure 26.** Western blot data showing the effect of sex on protein levels of myelin proteolipid protein (PLP) in the orbital frontal cortex (OFC) of adult rats. Mean density  $\times$  area (DxA) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for PLP and the loading control  $\beta$ -actin are shown in insets above the graph. Overall main effect of sex: \*  $p < 0.05$ .

*MBP in dSTR in adult rats*

Western blots revealed four isoform bands of MBP-like immunoreactivity at approximately 21.5, 18.5, 17.0, and 14.0 kDa. As illustrated in Figure 27, analysis of protein levels of MBP in the dSTR of male and female adult rats revealed a main effect of MBP isoform ( $F(3,48) = 72.46$ ,  $p < 0.001$ ), indicating that protein levels varied across the four isoforms. However, there was no main effect of sex or interaction between sex and MPB isoform. There was no main effect of sex on levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that expression of MBP in the dSTR is not significantly different between adult male and female rats.

**Figure 27**

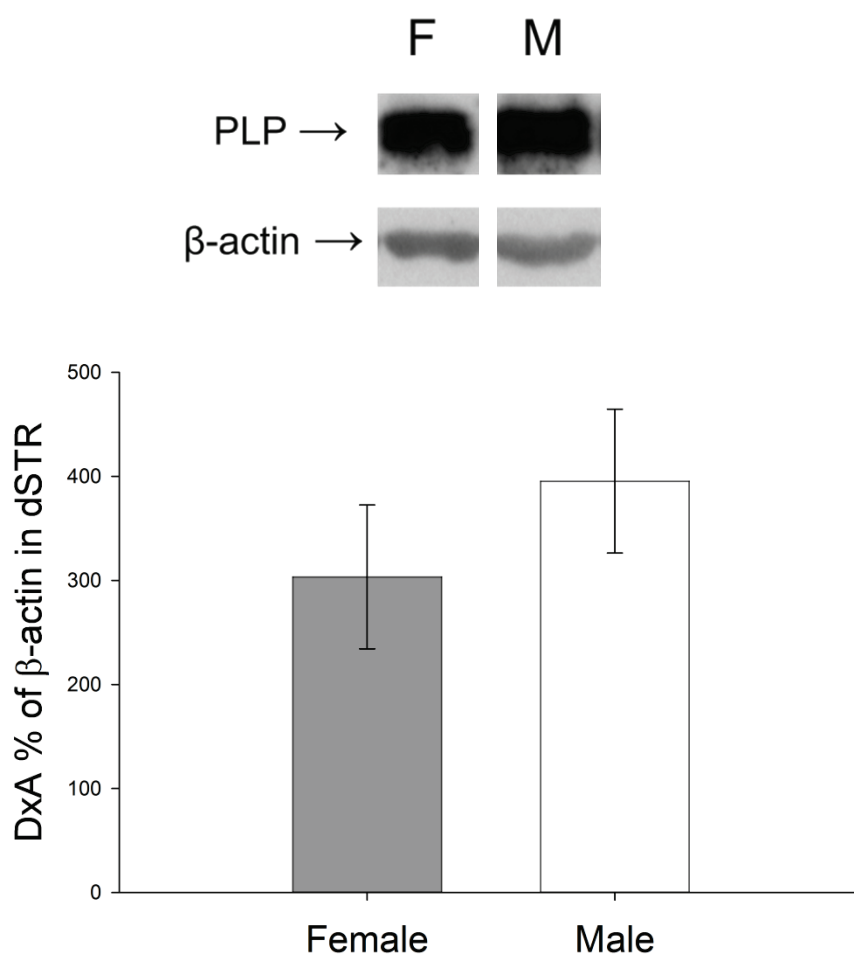
**Figure 27.** Western blot data showing the effect of sex on protein levels of myelin basic protein (MBP) with isoforms averaged (left) and isolated (right) in the dorsal striatum (dSTR) of adult rats. Mean density  $\times$  area ( $D \times A$ ) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for MBP and the loading control  $\beta$ -actin are shown in insets above the graph.

#### *PLP in dSTR in adult rats*

Western blots revealed a band of PLP-like immunoreactivity at approximately 23 kDa. As illustrated in Figure 28, analysis of protein levels of PLP in the dSTR of male and female adult rats revealed no main effect of sex. There was no main effect of sex on

levels of  $\beta$ -actin, indicating that equal amounts of total protein were loaded for all animals. Results indicate that expression of PLP in the dSTR is not significantly different between adult male and female rats.

**Figure 28**



**Figure 28.** Western blot data showing the effect of sex on protein levels of myelin proteolipid protein (PLP) in the dorsal striatum (dSTR) of adult rats. Mean density  $\times$  area (D $\times$ A) ( $\pm$ SEM) expressed relative to control  $\beta$ -actin protein levels. Representative blot images for PLP and the loading control  $\beta$ -actin are shown in insets above the graph.

## **Experiment 4b: Analysis of projections from the OFC to the dSTR in adult rats using an anterograde tracer**

### *Hypothesis*

I hypothesized that when an anterograde tracer was taken up by neurons in the OFC, females would have a greater expression of the tracer in the dSTR than would males, indicating that females have more projections from the OFC to the dSTR than do males.

## **Methods**

### *Subjects*

Eight male and eight female Long-Evans hooded rats, approximately 2 months of age, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed in same-sex pairs, and animal care was the same as described in Experiment 1.

### *Stereotaxic surgery and tracer injection*

A week after arrival, rats underwent stereotaxic surgery to allow for bilateral microinjection of the anterograde tracer, biotinylated dextran amine (BDA), into the OFC. BDA is a widely used anterograde tracer that is selectively taken up by neurons at the site of injection (Wang et al. 2013). BDA is well transported both retrogradely and anterogradely depending on its molecular weight (Lazarov 2013). At 10 kDa, as was used in the current experiment, BDA is anterogradely transported. BDA is biotinylated (Reiner et al. 2000). Thus, there is no need to attach a biotin-conjugated secondary to BDA in order to visualize a peroxidase reaction product after incubation with avidin biotin

peroxidase complex (ABC) followed by diaminobenzidine (DAB) during immunohistochemistry (Lazarov 2013). Anterograde tracer and immunohistochemistry techniques were based upon Springer Protocols for anterograde tracing experiments (Lazarov 2013; Wang et al. 2013).

Surgeries were conducted on anesthetized rats using appropriate aseptic techniques. Sterile gloves and drapes were used. Instruments were sterilized in an autoclave prior to surgeries. Between surgeries, instruments were cleaned with 70% ethanol and sterilized in a glass bead sterilizer. Rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). Buprenorphine (0.025 mg/kg, Buprenex) was administered subcutaneously as an analgesic. Depth of anesthesia was determined by tail pinch and by checking for reflexes by pulling the hind leg. For anterograde, a rat was placed in a stereotaxic apparatus. Incision sites were cleaned thoroughly with 70% ethanol followed by iodine-povidine solution. An incision was made along the midline of the head of the rat, and the underlying fascia was scraped to the side. The local anesthetic, lidocaine (0.5% no more than 7 mg/kg), was applied to the fascia at the time of the incision. Using a small drill mounted on a stereotaxic arm, a 0.9 diameter hole was drilled through the skull. Using a 10  $\mu$ l Hamilton syringe, 0.5  $\mu$ l per hemisphere of 10% BDA (10 kDa) in aCSF were infused bilaterally into the OFC (+3.2 mm anteroposterior,  $\pm$ 2.5 mm lateral, -4.6 mm dorsoventral; coordinates based on Paxinos Watson, 1998). The skull surface was completely dried. The entire infusion site was covered with sterile bone wax. Wound

closure was made with sterile absorbable sutures. Rats were monitored until fully awake from anesthesia before being returned to the vivarium.

#### *Perfusion and fixation*

After a survival period of 10 days that allowed for anterograde transport of the tracer to the dSTR, rats were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg). The chest and abdominal cavity was surgically opened. Rats were injected intracardially with 0.05 ml of Heparin, an anticoagulant. Rats were then perfused intracardially with 200 ml of 0.9% saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). After perfusion, brains were removed and post-fixed in a fresh fixative of 30% sucrose in 0.1 M PBS.

#### *Tissue sectioning*

Frozen coronal sections (50  $\mu$ m each) of the dSTR (from +1.7 mm to -0.4 mm anteroposterior) were collected in cyroprotectant (1% polyvinylpyrrolidone, 30% sucrose, 30% ethylene glycol in 0.1 M PBS) using a sliding microtome. Sections were store in 0.5 ml vials in cyroprotectant at 4°C until further use. Three consecutive sections at +0.5 mm anteroposterior were used for imaging and statistical analysis of BDA expression.

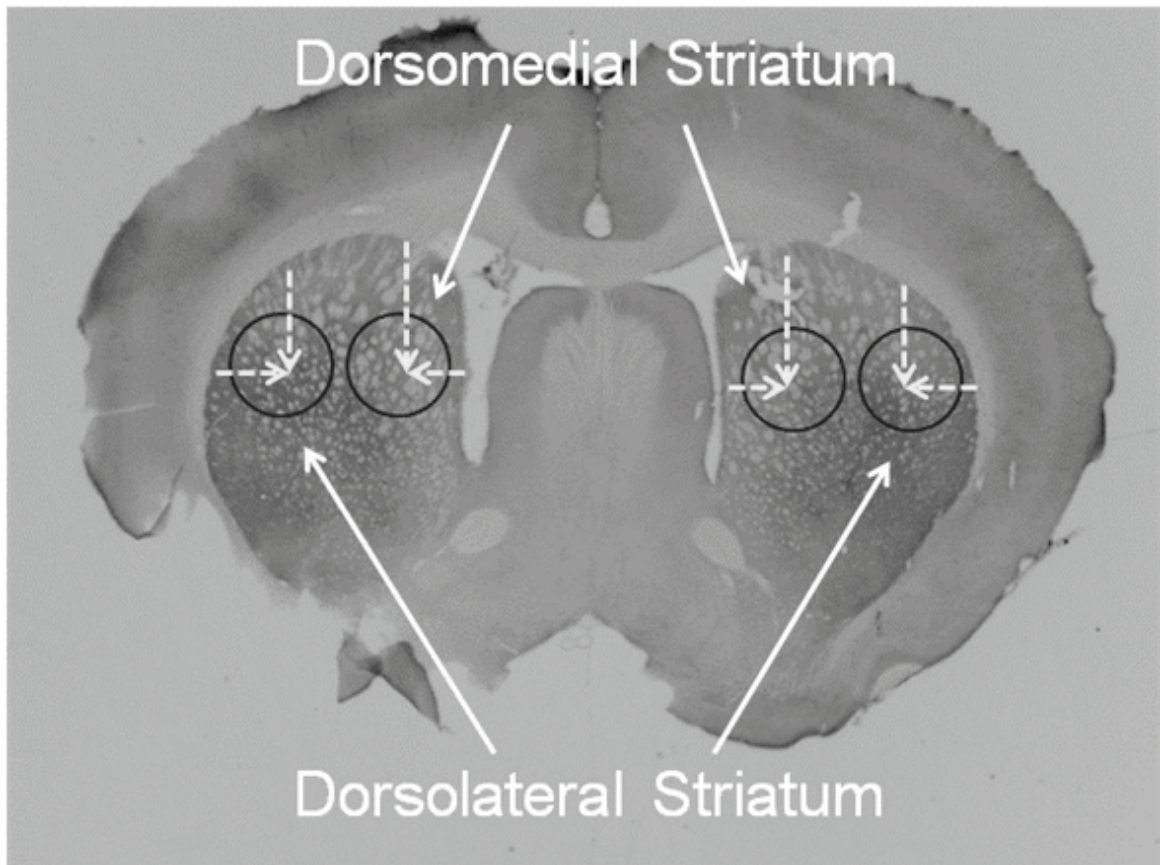
#### *Immunohistochemistry*

Sections were washed three times for 10 min each in 0.05M PBS and then incubated for 60 min in 0.1% bovine serum albumin in 0.05M PBS to block endogenous peroxidases. Following blocking, sections were washed three times for 10 min each in

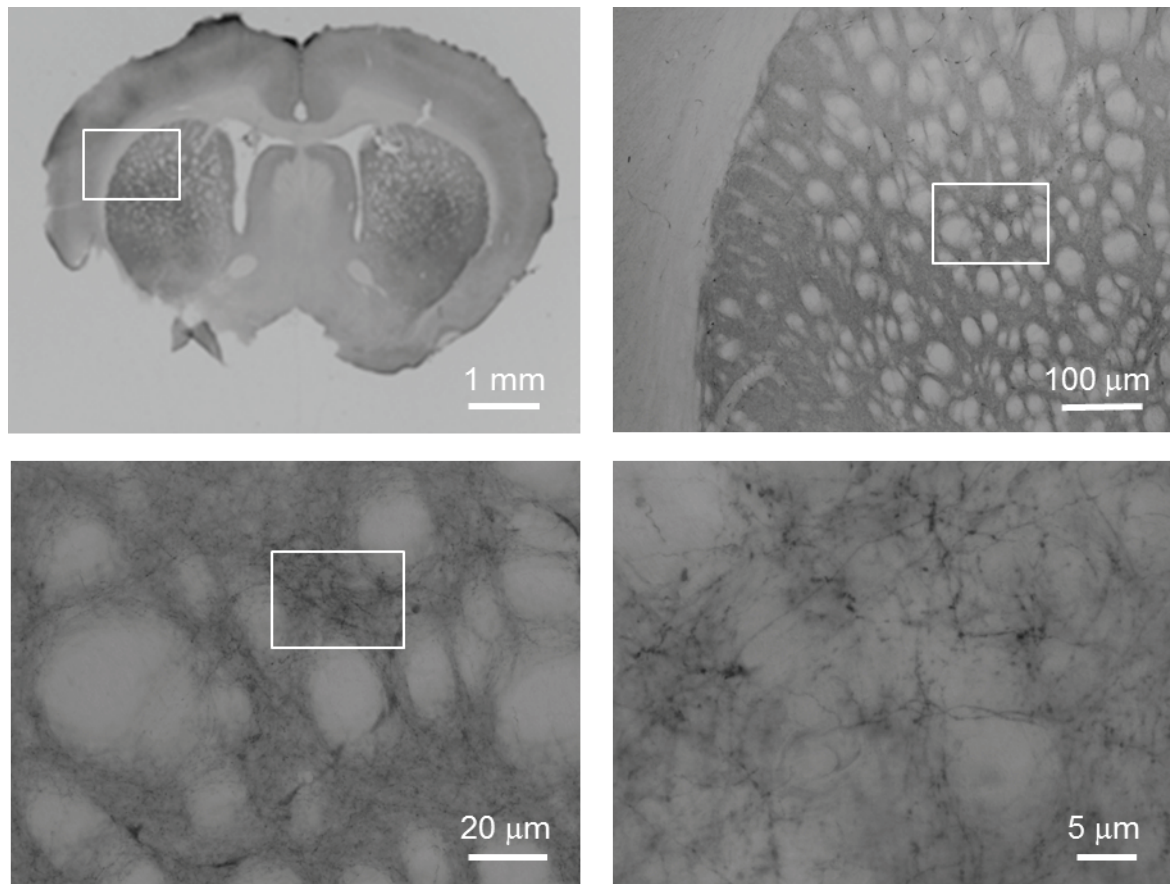
0.05M PBS. Sections were then incubated for 120 mins in avidin biotin peroxidase complex (ABC) reagent (Vectastain Elite Kit, Vector Laboratories, Inc.) in 0.3% Triton X in 0.05M PBS. Following three more washes for 10 min each in 0.05M PBS, the peroxidase reaction product was visualized by incubation in a solution containing 0.05% diaminobenzidine (DAB; Sigma Aldrich) and 0.01%  $\text{H}_2\text{O}_2$  in 0.1M PBS for 7 min. Sections were then wash again three times for 10 min in 0.01M PBS, mounted onto gelatin-coated slides, and allowed to air-dry overnight. The following day, the slides were placed in xylene for 5 min, and then coverslipped using diluted permount.

#### *Imaging and quantification of BDA expression*

Sections were imaged at 20x magnification using a light microscope (Olympus IX71) and digital camera (Hamamatsu Camera; 0.1 s exposure) interfaced with HC Image Software. MCID Analysis software was used to calibrate images to control for variations in background density. Optical density was measured within circular regions (1 mm diameter) of the dorsolateral striatum (dlSTR) determined by measuring 0.6 mm horizontally and 0.75 mm vertically from the corpus callosum and within circular regions (1 mm diameter) of the dorsomedial striatum (dmSTR) determined by measuring 0.6 mm horizontally from the lateral ventricle and 1 mm vertically from the corpus callosum (Figure 29). Figure 30 provides example images of anterograde tracer staining in the dSTR at varying magnification levels.

**Figure 29**

**Figure 29.** Defined areas of measurement within the dorsolateral striatum and dorsomedial striatum. Circles represent 1 mm diameter circular regions of analysis.

**Figure 30****Figure 30.** Anterograde tracer staining in the dSTR at varying magnification levels.

### *Statistical analyses*

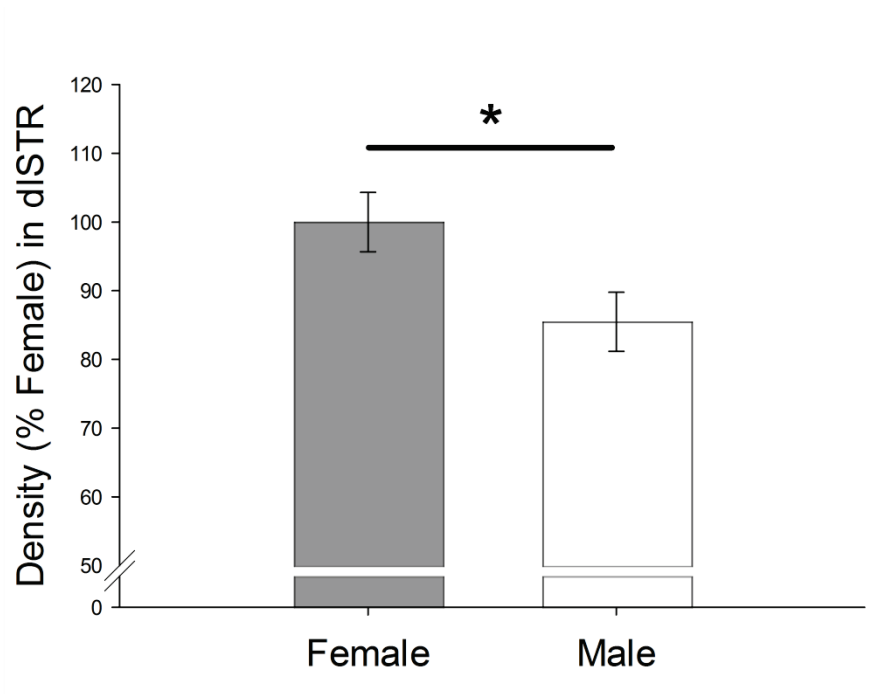
Three consecutive sections at +0.5 mm anteroposterior for each rat were imaged and optical density values were averaged for statistical analysis of BDA expression. The optical density values as a percentage of average female density for the left and right hemispheres of the dISTR and dmSTR were analyzed using an overall repeated measures ANOVA with hemisphere as the within-subjects factor and sex as the between-subjects factor for each brain region.

## **Results**

Brain tissue from two males and two females was damaged in the process of collection and analysis, resulting in the following final group numbers: male ( $n = 6$ ), female ( $n = 6$ ).

### *Anterograde tracer expression in dISTR*

Analysis of optical density of OFC-injected anterograde tracer in the dISTR of adult male and female rats revealed no significant main effect of hemisphere, indicating that the expression of the tracer was similar in the left and right hemispheres of the dISTR. As illustrated in Figure 31, there was a significant main effect of sex ( $F(1,10) = 5.67$ ,  $p = 0.039$ ), indicating that adult female rats have more projections from the OFC to the dISTR than do adult male rats.

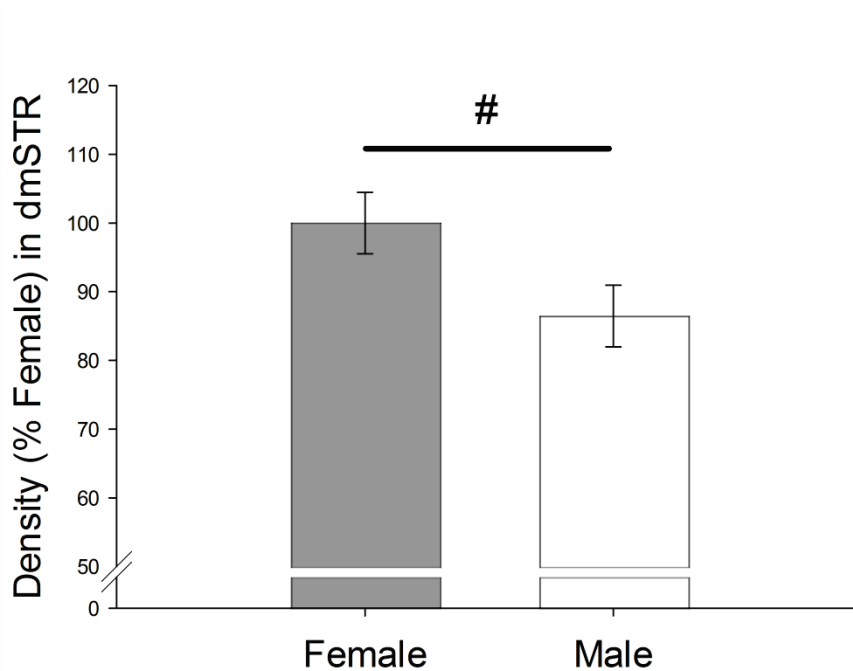
**Figure 31**

**Figure 31.** Density of anterograde tracer expression in the dorsolateral striatum (dlSTR) of adult male and female rats across hemispheres. \*  $p = 0.039$

### *Anterograde tracer expression in dmSTR*

Analysis of optical density of OFC-injected anterograde tracer in the dmSTR of adult male and female rats revealed no significant main effect of hemisphere, indicating that the expression of the tracer was similar in the left and right hemispheres of the dmSTR. As illustrated in Figure 32, there was a trend to significance for a main effect of sex ( $F(1,10) = 4.57$ ,  $p = 0.058$ ), suggesting that adult female rats have more projections from the OFC to the dmSTR than do adult male rats.

**Figure 32**



**Figure 32.** Density of anterograde tracer expression in the dorsomedial striatum (dmSTR) of adult male and female rats across hemispheres. <sup>#</sup>  $p = 0.058$

### **Experiment 4c: Effect of inactivation of the OFC on performance during a simplified operant test of impulsive action in adult rats**

#### *Hypothesis*

A simplified version of the 5-CSRTT with only one stimulus location was used to isolate impulsive action and focus on the inhibition of a habitual striatal response. I hypothesized that if a sex difference in impulsive action is the result of females having greater OFC control over the dSTR than males, then the inactivation of the OFC should decrease female impulsive action levels to that of males, eliminating the sex difference in impulsive action.

#### **Methods**

##### *Subjects*

Four male and four female Long-Evans hooded rats, approximately 2 months of age, were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed in same-sex pairs, and animal care was the same as described in Experiment 1. All animals were weighed daily following behavioral training and food was provided in their home cages to maintain their weights at 85% of their free-feeding weights while allowing for growth of approximately 2% of their body weight each week. Initial plans were to add another replicate of animals to the study in order to raise the number of animals in the experiment to a large enough number for adequate comparison of performance between the sexes. However, after noticing the apparent lack of a lidocaine induced effect on performance, we opted to not conduct any unnecessary

surgical procedures on any additional animals and did not add another replicate to the study.

### *Apparatus*

Animals were trained and tested in the operant chambers described in Experiment 2b. In order to simplify the task and focus on testing the inhibition of an impulsive habitual response, only one hole on the rear wall was active. Each animal received one session of training per day throughout the experiment. House lights were on unless stated otherwise.

### *Behavioral training*

Animals were trained daily for 30 min while passing through the same training stages as in Experiment 2b. An animal moved to the next training stage once it performed 100 trials at <20 percent omissions on a single session, as compared to two consecutive sessions in Experiment 2b. Because there was only one light aperture, percent correct was not relevant. For the final training stage (baseline training), the cue duration was reduced to 1 sec, as compared to 0.5 sec in Experiment 2b. Training with this protocol continued until animals perform 100 trials at a criterion of <20 percent omissions for five consecutive days.

### *Vaginal cytology*

To control for effects of fluctuating ovarian hormones on performance, vaginal smears of female rats were collected daily as in Experiment 2b. Behavioral testing was only administered when a female was at the proestrous stage of the estrous cycle, at which time estradiol levels are at their peak and vaginal cytology is characterized by

large nucleated epithelial cells (Becker et al. 2005). Each male was paired with a particular female and was always tested at the same time as that female.

### *Stereotaxic surgeries*

Following initial testing, rats underwent stereotaxic surgery to implant cannula to allow for temporary inactivation by lidocaine of the OFC during testing. Surgeries were conducted on anesthetized rats using appropriate aseptic techniques as described in Experiment 4b. Bilateral guide cannulas (Plastics One, Roanoke, VA) were anchored to the skull with dental acrylic. Guide cannula (28 gauge) were aimed at the OFC (+3.2 mm anteroposterior,  $\pm 2.5$  mm lateral,  $-4.6$  mm dorsoventral; coordinates based on Paxinos Watson, 1998). The skull surface was completely dried. The entire cannulation site was covered with dental cement. The cement was allowed to harden. Wound closure was made with sterile absorbable sutures. Dust caps (Plastics One), which screwed onto the threaded pedestals, were used for protection when cannulas were not in use. Rats were monitored until fully awake from anesthesia before being returned to the vivarium. Rats were allowed one week for recovery.

### *Behavioral testing*

Rats were retrained on baseline training until animals perform 100 trials at a criterion of <20 percent omissions for five consecutive days. Rats continued to be trained under baseline conditions until proestrus occurred. During baseline training, the stimulus lasted for 1 sec. A 5 sec ITI was presented before the onset of stimulus. Rats were given 5 sec to respond before an omission was counted. Each session consisted of 100 trials.

To challenge the ability of the rats to inhibit an impulsive habitual response, an Unpredictable Long ITI condition, in which the time before the onset of the light stimulus was pseudorandomly lengthened to 5.0, 7.5, 10.0, or 12.5 sec distributed across the 100 trials, was introduced for one daily session on the first day of proestrus following completion of baseline training. Fifteen minutes prior to this testing period, half of the rats received intra-OFC infusion of lidocaine and half received intra-OFC infusion of aCSF vehicle. With the exception of the change in ITI duration, all other parameters were the same as baseline during the Unpredictable Long ITI condition. Rats then received 5 days of baseline training in order to retrain the rats on a predictable stimulus. Following completion of this baseline training, another Unpredictable Long ITI condition was introduced for one daily session on the next day of proestrus. Fifteen minutes prior to this testing period, half of the rats received intra-OFC infusion of lidocaine and half received intra-OFC infusion of aCSF vehicle. With the exception of percent correct, the same behavioral measures as in Experiment 2b (premature responses, perseverative responses, percent omissions, response latency, and reward latency) were measured.

#### *Microinfusion procedure*

Fifteen minutes prior to testing on the Unpredictable Long ITI condition, rats received bilateral infusions to the OFC of either 0.5  $\mu$ l of 20% lidocaine hydrochloride solution (200 mg/ml) or aCSF vehicle counterbalanced across the two testing periods (Kantak et al. 2009; Packard and McGaugh 1996). The total volume of 0.5  $\mu$ l resulted in a lidocaine dose of 100  $\mu$ g that was infused bilaterally at a rate of 0.50  $\mu$ l/min. The

injection cannula (30 gauge) extended 1.75mm beyond the tip of the guide cannula and was connected to an assembly that consisted of a polyethylene supply tube encased in tough vinyl tubing with a captive collar to secure the unit to the cannula. When tightened, the cap hydrostatically sealed the supply tube to the cannula permitting injection while the animal moved freely in its cage. A microinfusion pump (Harvard Apparatus) was used to control the volume and rate of infusion, which was delivered over a period of 2 minutes. The cannula was left in place for an additional two minutes to allow for diffusion. Following each procedure, the integrity of the cannula was determined by visual inspection. Dust caps were reapplied to protect the cannula when not in use.

#### *Cannula placement confirmation*

Following completion of behavioral testing, animals were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) and killed by decapitation. Whole brains were removed, quick-frozen on dry ice, and stored at -80°C. In a cryostat at -20°C, frozen coronal sections (20 µm) were taken from the OFC of each brain, thaw mounted onto gelatinized slides, stained with 0.5% cresyl violet, and microscopically examined for verification of cannula placement.

#### *Statistical analyses*

To assess the stability of baseline performance at the end of training, performances across the last five days of training were analyzed for all dependent variables using overall repeated measures ANOVAs with day as the within-subjects factor and sex as the between-subjects factor. To assess any possible changes in

baseline performance between drug and vehicle Unpredictable Long ITI testing periods, performances across the initial baseline data and baseline data collected the day before each Unpredictable Long ITI condition were analyzed for all dependent variables using overall repeated measures ANOVAs with day as the within-subjects factor and sex as the between-subjects factor. To assess performance during the drug and vehicle Unpredictable Long ITI testing periods, performances during the drug and vehicle Unpredictable Long ITI testing days were analyzed using an overall repeated measures ANOVA with drug treatment as the within-subjects factor and sex as the between-subjects factor for all dependent variables.

## **Results**

### ***Baseline training***

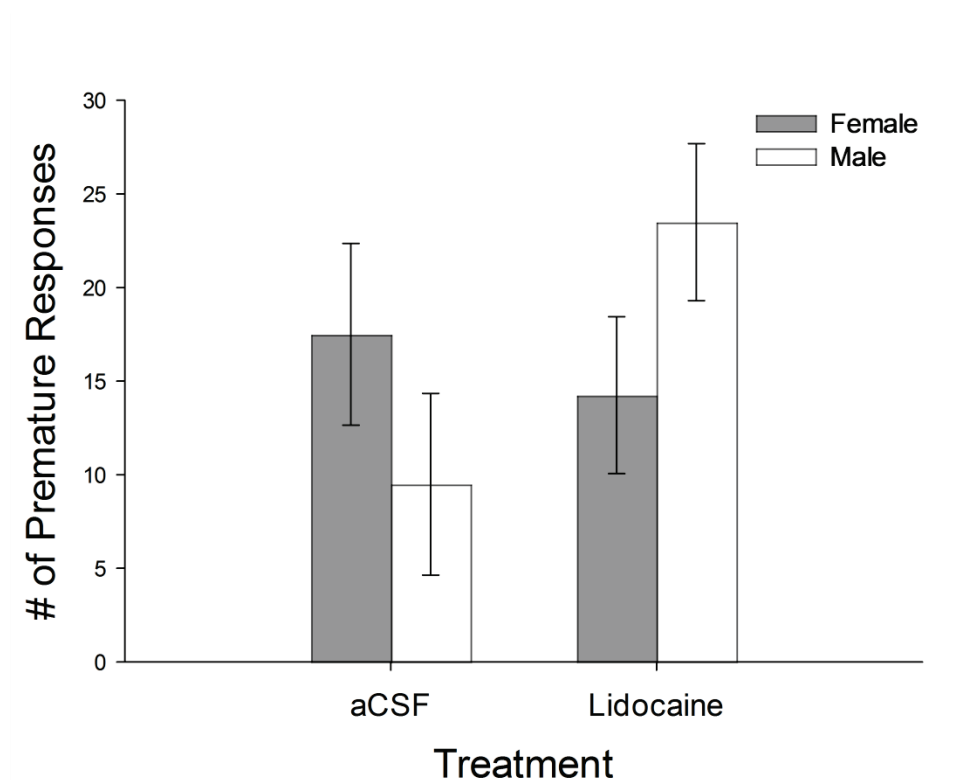
Performance remained stable across the last five days of baseline training, and there were no significant sex differences on any measure during the last five days of training. In addition, no significant change in baseline performance occurred across baseline sessions conducted between the drug and vehicle testing periods. There was no main effect of sex on the two measures of speed, correct response latency and reward collection latency, indicating that there was no sex difference in motor function, sensory function, motivational factors, or the overall ability of the animals to perform the task (Robbins 2002).

### ***Drug and vehicle behavioral testing***

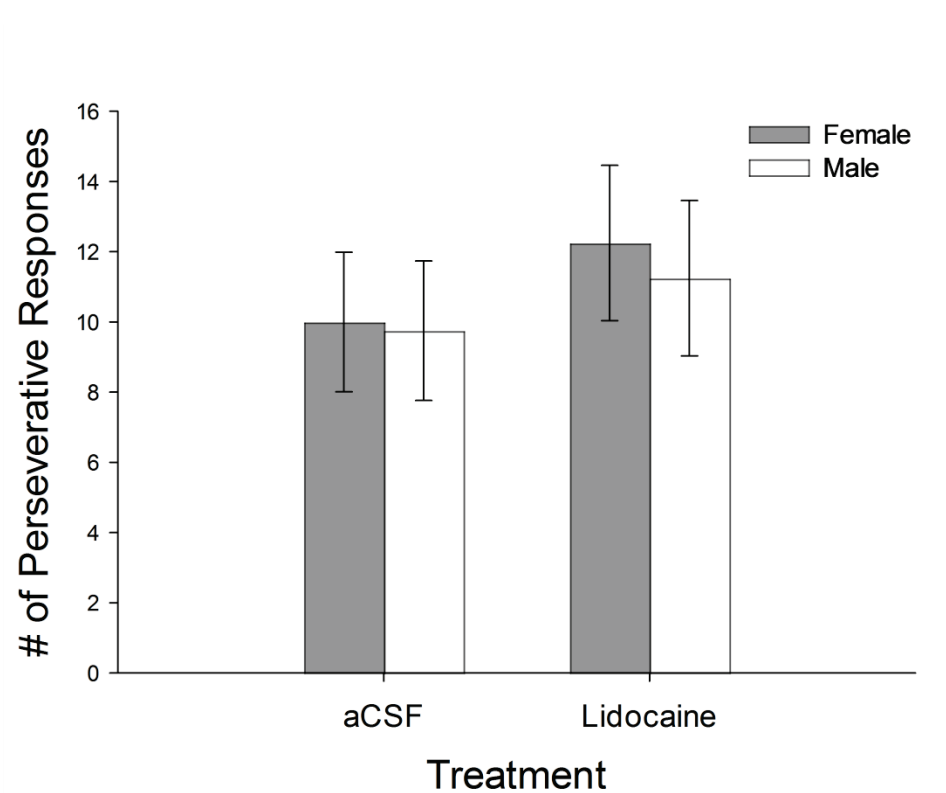
#### *Measures of impulsive action*

As illustrated in Figures 33 and 34, analysis of the number of premature responses and the number of perseverative responses in adult male and female rats revealed no main effects of drug treatment, sex, or interactions between drug treatment and sex, indicating that impulsive action was not disrupted by infusion of lidocaine into the OFC during the Unpredictable Long ITI conditions in either sex.

**Figure 33**



**Figure 33.** Number of premature responses in adult male and female rats following infusion of either aCSF vehicle or lidocaine into the orbital frontal cortex during Unpredictable Long ITI testing conditions.

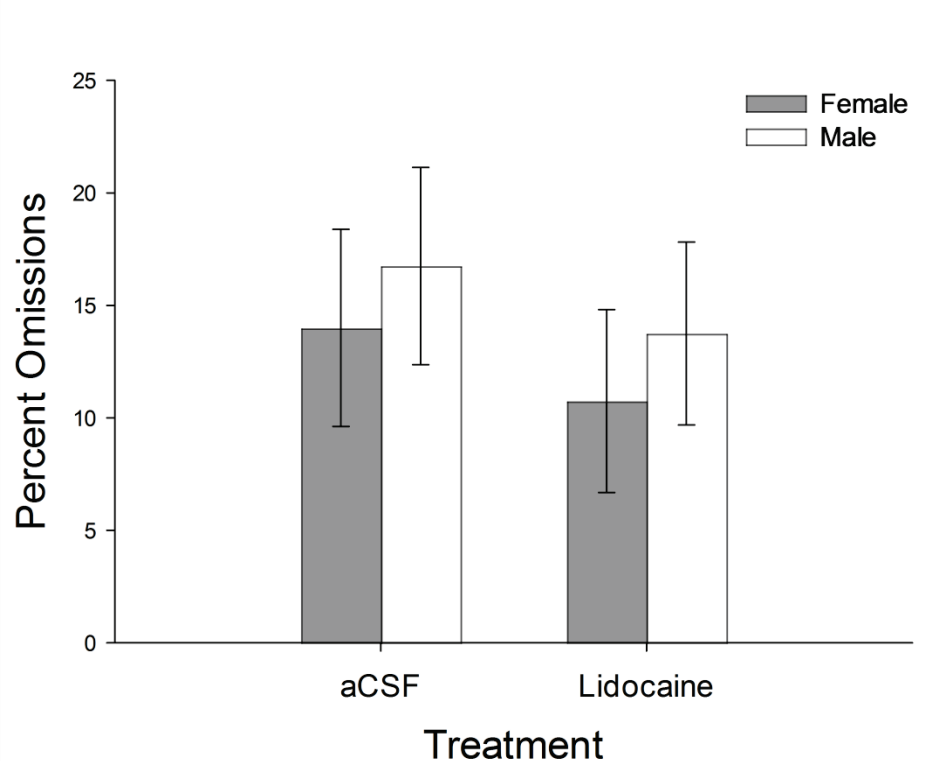
**Figure 34**

**Figure 34.** Number of perseverative responses in adult male and female rats following infusion of either aCSF vehicle or lidocaine into the orbital frontal cortex during Unpredictable Long ITI testing conditions.

*Measure of attentional performance*

As illustrated in Figure 35, analysis of percent omissions in adult male and female rats revealed no main effect of treatment, sex, or interaction between treatment and sex, indicating that attentional performance was not disrupted by infusion of lidocaine into the OFC in either sex.

**Figure 35**



**Figure 35.** Percent omissions in adult male and female rats following infusion of either aCSF vehicle or lidocaine into the orbital frontal cortex during Unpredictable Long ITI testing conditions.

### *Speed measures*

As shown in Table 2, on the two measures of speed, response latency and reward collection latency, there were no significant main effects of treatment, sex, or interactions between treatment and sex, indicating that there were no sex differences or effect of lidocaine-induced inactivation of the OFC on motor function, sensory function, motivational factors, or the overall ability of the animals to perform the task during the behavioral challenges (Robbins 2002).

**Table 2**

**Table 2**

Reward collection latency and response latency following infusion of either aCSF or lidocaine into the orbitofrontal cortex (OFC)

	aCSF		Lidocaine	
	M	S.E.M.	M	S.E.M.
<b>Response latency (sec)</b>				
<b>Female</b>	0.63	0.06	0.62	0.05
<b>Male</b>	0.58	0.06	0.60	0.05
<b>Reward latency (sec)</b>				
<b>Female</b>	1.05	0.05	1.08	0.04
<b>Male</b>	1.05	0.05	1.01	0.04

### **Discussion**

Collectively, the present experiments demonstrate for the first time that there is a sex difference in impulsive choice control in prepubertal rats that is organized neonatally by the actions of both androgens and estrogens, this sex difference subsides in adulthood, but a sex difference in impulsive action control is present in adulthood. Furthermore, we demonstrated that adult female rats have increased levels of myelination within the OFC and increased strength of projections from the OFC to the dSTR as compared to males establishing a potential molecular mechanism that could underlie the enhanced impulse control in females as compared to males.

Prepubertal rats exposed to treatments that resulted in neonatal androgenic, but not estrogenic, effects (DHT-treated females and formestane-treated males) or estrogenic, but not androgenic, effects (estradiol-treated females) made more impulsive choices than did control females, and their performance mirrored that of control males. Assessment of impulsive choice responding in adult rats indicated no differences between the sexes. However, assessment of impulsive action responding in adult rats revealed that males made more impulsive actions than did females. Protein levels of DAT and NET in the OFC and dSTR were similar between the sexes in prepubertal and adult rats, indicating that there were no differences in dopaminergic or noradrenergic reuptake in the OFC or dSTR between the sexes. Protein levels of MBP and PLP protein in the OFC and dSTR were similar between the sexes in prepubertal rats but increased in the OFC but not dSTR in adult female rats as compared to males, indicating that myelination and neural connectivity is greater in the OFC but similar in the dSTR in

females as compared to males. Furthermore, assessment of the strength of projections from the OFC to the dSTR using an anterograde tracer injected into the OFC and measured in the dSTR discovered that adult female rats had more projections from the OFC to the dSTR than did adult male rats. However, inactivation of the OFC during an impulsive action task in adult rats failed to have an effect on impulsive action responding in males and females. The novel discovery that adult female rats have increased levels of myelination within the OFC and increased strength of projections from the OFC to the dSTR as compared to males establishes a molecular sex difference that could possibly underlie the enhanced impulse control in females.

### **Neonatal hormones and impulsive choice**

Preliminary studies established that neonatal testosterone exposure was able to masculinize and increase impulsive behavior in prepubertal female rats. In Experiment 1, to determine the mechanism by which neonatal testosterone exerts its masculinizing effects on impulsive behavior, male rats were treated neonatally with control vehicle or an aromatase inhibitor to block the conversion of testosterone to estradiol (neonatal activation of androgen, but not estrogen, receptors). Female rats were treated neonatally with control vehicle, estradiol (neonatal activation of estrogen, but not androgen, receptors), or the non-aromatizable androgen dihydrotestosterone (neonatal activation of androgen, but not estrogen, receptors). I hypothesized that neonatal activation of estrogen receptors but not androgen receptors would masculinize impulsive choice behavior in prepubertal female rats. Results demonstrated that when

given the choice between an immediate small food reward and a delayed large food reward, prepubertal male rats made more impulsive choices (the selection of the immediate small reward) than did female rats. Like control males, prepubertal rats exposed to treatments that resulted in neonatal androgenic, but not estrogenic, effects (DHT-treated females and formestane-treated males) or estrogenic, but not androgenic, effects (estradiol-treated females) all made more impulsive choices than did control females. These findings extend the organizational impacts of gonadal hormones to a PFC-dependent behavior and demonstrate that neonatal actions of estrogens or androgens are sufficient to masculinize impulsive behavior in prepubertal rats.

These results are slightly different from my initial hypothesis that only estrogen receptor activation would result in masculinization of impulsive behavior. Estrogen receptor activation did masculinize impulsive choice behavior, but so did androgen receptor activation. Most research studies into the effects of early life testosterone exposure on behavior have focused on hypothalamic-dependent behaviors as the hypothalamus is highly sexually dimorphic (Sakuma 2009). In the hypothalamus, the effects of neonatal testosterone are dependent upon its conversion into estradiol by the enzyme aromatase once inside the cell (Gorski 1993). Neonatal administration of estradiol, but not DHT, masculinizes and defeminizes hypothalamic-dependent sexual behaviors in rodents (For review see, Lenz and McCarthy 2010). Our results indicate that androgens are able to masculinize PFC-dependent behavior. These results are consistent with previous data indicating that some brain areas outside of the hypothalamus are masculinized by androgens. For example, neonatal administration of DHT has been

shown to masculinize amygdala-dependent social behaviors (Bodo and Rissman 2008). Furthermore, in another sexually dimorphic brain area, the bed nucleus of the stria terminalis (BNST), it was reported that the size of the sex difference in cell number in this brain area was reduced in testicular-feminized mutant (Tfm) male mice that express hypofunctional androgen receptors, suggesting that neonatal androgen receptor activation is important for the development of sex differences in this brain area (Durazzo et al. 2007). Our finding that both neonatal estrogens and androgens affect impulsive choice responding suggests that the neonatal effects of androgens and estrogens may result in similar developmental changes in the brain that results in similar impulsive choice behavior later in life. However, it is also possible that neonatal androgenic and estrogenic effects could cause different developmental changes in the brain but the end result of these changes is a similar set of phenotypic impulsive choice behaviors later in life. Future studies are needed to make this distinction.

The mechanisms by which estrogens or androgens are able to alter neurodevelopment during the critical neonatal period to cause sex differences in impulsive behavior later in life remain unknown but may involve sex-specific epigenetic changes to the chromatin. Estrogens and androgens exert their neonatal effects by binding to their respective intracellular receptors (McCarthy 1994). Estrogen and androgen receptors are transcription factors that bind to hormone-responsive elements in the promoter regions of target genes and alter the rate of gene transcription (Beato and Sanchez-Pacheco 1996). When bound to their response elements, steroid receptors recruit coregulator proteins, such as histone acetyltransferase (HAT) and histone

methyltransferase (HMT), which add acetyl and methyl groups, respectively, to the N-terminal of histone tails (McKenna et al. 1999). Histone acetylation causes changes to the chromatin that provides transcriptional machinery with greater access to target genes leading to increased gene transcription (Turner 2000). Histone methylation causes changes to the chromatin that can have repressing or enhancing effects on gene transcription depending upon the location of methylation on the histone tail (Goll and Bestor 2002). These epigenetic changes to the chromatin induced by estrogens and androgens during the critical period of neonatal development can have long lasting effects (Gagnidze et al. 2010) and may underlie the sex differences in impulsive choice behavior in prepubertal rats reported here. Future studies are needed to determine if this is the case.

### **Impulsive choice vs. impulsive action**

To determine if the sex difference in impulsivity seen prior to puberty continues into adulthood, impulsive choice and action levels of adult male and female rats were assessed in Experiment 2. I hypothesized that when the demands on impulse control were increased, a sex difference in impulsive choice responding would be discovered, such that adult female rats would make fewer impulsive choices than would adult male rats and that similar to impulsive choice responding, adult female rats would make fewer impulsive actions than would adult male rats on an impulsive action task. Results indicated that adult males and females exhibited similar levels of impulsive choice behavior during performance of the delay-based impulsive choice task. However,

assessment of impulsive action responding during the 5-CSRTT revealed that adult males made more impulsive actions than did females.

Preliminary studies indicated that there is no sex difference in impulsive choice responding in adult rats. However, it was unclear whether or not the delays used in that study were long enough to reliably measure a difference in performance in adults, which were not as affected by the delay lengths used in prepubertal rats. In Experiment 2a, when adult rats were tested on the delay-based impulsive choice task under increased delay conditions, males and females still chose an immediate small food reward over a delayed large food reward at similar levels. These results indicate that the sex difference in impulsive choice behavior subsides after puberty.

In Experiment 2b, the number of impulsive actions, as measured by the number of premature responses, was greater in adult male rats than it was in females when a delayed response was required. Males committed more premature responses than did females when the onset of the stimulus was unpredictably lengthened. There were no differences in impulsive action behavior under baseline conditions. Only when the system was challenged by increasing task difficulty were sex differences in impulsive action behavior revealed. Interestingly, it was also discovered that attentional performance, as measured by percent correct and number of omissions, was more disrupted in female rats than it was in male rats during the behavioral challenges. Female rats displayed a greater decrease in percent correct when the onset of stimulus was unpredictably lengthened and a greater increase in omitted responses throughout the behavioral challenges as compared to performance in males. There were no sex

differences in reward collection latency or correct response latency, indicating that omissions were the result of inattentiveness and not motivational or appetitive factors.

The sex differences in impulsive action and attentional performance were most prominent when the onset of the stimulus was unpredictably lengthened. During the Unpredictable Long ITI behavioral challenge, animals experience both a change in the duration of time between events and a change in the predictability of events. Percent correct did not change across the different ITI durations. Therefore, the impaired attentional performance in female rats as compared to male rats is most likely due to the unpredictability of the stimuli rather than the increased ITI duration. Premature responding varied across the different ITI durations, and males made more premature responses than did females during the longer ITI trials. Therefore, the impaired impulse control in male rats as compared to female rats is most likely due to the increased ITI duration rather than the unpredictability of the stimuli. Thus, adult male rats outperform adult female rats when attentional resources must be continuously allocated in order to detect rare events when stimuli are presented unpredictably in time, and adult female rats are better at refraining from making premature responses than are adult male rats when the duration of time between stimuli is increased.

In Experiment 2a, we failed to find a sex difference in impulsive responding in adult rats during performance of the delay-based impulsive choice task, whereas in Experiment 2b, we did discover a sex difference in impulsive responding in adult rats during performance of the 5-CSRTT such that males exhibited greater impulsivity than did females when a delayed response was required. As discussed in the Introduction,

premature responding in the 5-CSRTT and impulsive choice in the delay-based impulsive choice task are mediated by different cognitive processes, impulsive action and impulsive choice, respectively. In addition, the 5-CSRTT may be a more sensitive measure of inhibitory control than is the delay-based impulsive choice task. A session in the 5-CSRTT is comprised of 100 trials, whereas in the delay discounting task only 10 trials were used from each delay condition. The disappearance of a sex difference in impulsive choice in adulthood could also be due to the maturation of the brain with age. Levels of inhibitory control do increase with age (Moilanen et al. 2009), and thirteen healthy children for whom anatomic brain MRI scans were obtained every 2 years for 8–10 years revealed that the frontal cortex is not fully developed until around 18 years of age (Gogtay et al. 2004).

Changes in impulsive behavior in adulthood could also be caused by the activational effects of hormones. Estrogens and androgens affect behavior and physiology through two distinct mechanisms: early-life organizational effects that produce generally permanent changes in brain morphology that influence male and female behavior in adulthood and post-pubertal activational effects that act on the fully developed nervous system to produce or maintain male and female behavior in adulthood (Nelson 2005a). Our lab has demonstrated the activational effect of estradiol on improvement in performance on the 5-CSRTT in female rats (Bohacek and Daniel 2010). Interestingly, estradiol improved attentional performance during the behavioral challenges in which the stimuli are presented unpredictably in time, the same behavioral challenges in which sex differences were seen in the current study. However,

estradiol had no effect on impulsive action behavior in that study. During assessment of impulsive action behavior in the current study, we controlled for fluctuations in estradiol levels by always testing females during the proestrous stage of the estrous cycle, at which time estradiol levels are at their peak (Becker et al. 2005). Therefore, we cannot infer any information about the activational effects of estradiol on impulsive action behavior as estradiol levels were invariable throughout testing. During assessment of impulsive choice behavior, we tracked the stages of the estrous cycles in the female rats, but due to the design of the behavioral testing, the day of proestrus fell during different delay conditions across animals causing the sample sizes for each stage of the estrus cycle to be very small. No differences in impulsive choice behavior were observed across the estrous cycle, suggesting that estradiol levels did not affect impulsive choice behavior in adult female rats.

The present findings expand upon the current knowledge about sex differences in impulsive action and attentional processes in rats. Previous results using simplified versions of the 5-CSRTT, in which stimuli are presented for longer durations from only two locations, have found mixed results. Results of one study are consistent with our findings of a female advantage in impulsive action control and a male advantage in attentional performance (Jentsch and Taylor 2003). However, another study reported little or no sex difference in impulsive action control or attentional performance (Burton and Fletcher 2012). In the current study, we only found sex differences in impulsive action control and attentional performance under challenging conditions. The current study is the first to examine sex differences in impulsive action and attentional

performance using the extensive training protocol of the 5-CSRTT. Animals received up to 70 training sessions until they performed at >70% correct and <20% omissions on baseline training for five consecutive days. Our results demonstrate that a female advantage in impulsive action control and a male advantage in attentional performance are present under challenging conditions during which the time before the onset of the stimulus is unpredictably lengthened.

### **Dopamine, norepinephrine, and impulse control**

To determine if differences in dopaminergic and noradrenergic functioning might underlie the enhanced impulse control seen in females, protein levels of DAT and NET in the OFC and dSTR of prepubertal and adult rats were measured in Experiment 3. I hypothesized that in prepubertal rats, control female rats would have increased levels of DAT and NET in the OFC and dSTR as compared to control males and females treated with neonatal testosterone, indicating greater dopaminergic and noradrenergic reuptake, which might suggest increased dopaminergic and noradrenergic neurotransmission. I also hypothesized that a similar sex difference would be seen in adult male and female rats. Analysis of protein expression of DAT and NET in the OFC and dSTR did not reveal any differences in expression levels between prepubertal or adult male and female rats. Increased levels of dopamine and norepinephrine result in the up-regulation of their respective transporters, DAT and NET (Furman et al. 2009; Sager and Torres 2011). Therefore, increased protein expression of DAT and NET should indicate increased signaling of the neurotransmitters that they flux. The results of

Experiment 3 suggest that the levels of dopamine and norepinephrine in the OFC and dSTR are similar in adult male and female rats. However, future studies directly measuring levels of dopamine and norepinephrine in the OFC and dSTR of adult rats must be conducted to fully support this conclusion. In addition, even if levels of dopamine and norepinephrine in the OFC and dSTR are equal in males and females, sex differences in receptor subtype levels or sensitivity could contribute to sex differences in impulsive behavior as dopamine and norepinephrine receptor subtypes can have opposing effects. For example,  $D_1$  and  $\beta_1$  receptors are excitatory and  $D_2$  and  $\alpha_2$  receptors are inhibitory (Meyer and Quenzer 2005). Our results are counter to what has been reported in humans. Women are reported to have higher dopamine  $D_2$  receptor binding in the frontal cortex (Kaasinen et al. 2001) and higher dopamine levels (Laakso et al. 2002) and expression of the dopamine transporter in the striatum as compared to men (Lavalaye et al. 2000).

Although, we failed to discover any significant differences between in the sexes in DAT or NET expression in the OFC or dSTR, both dopamine and norepinephrine affect impulsive responding. Our findings simply suggest that there is no difference between the sexes in the levels of the transporters for dopamine and norepinephrine in the OFC and dSTR in prepubertal and adult rats. Studies examining the effects of pharmacologically blocking the dopamine transporter thereby increasing dopamine in the PFC have reported both a decrease in impulsive choice responding (Richards et al. 1999; Sagvolden et al. 1992; Wade et al. 2000) and an increase in impulsive choice responding (Charrier and Thiebot 1996; Evenden and Ryan 1996). In addition,

atomoxetine, which selectively blocks the norepinephrine transporter, decreases impulsive choice responding during the delay-based impulsive choice task (Robinson et al. 2008).

### **Orbital frontal cortex, dorsal striatum, and impulse control**

In Experiment 4, three studies were conducted to determine if greater OFC control over the dSTR in females as compared to males provides females with a greater ability than males to inhibit undesirable behaviors. To determine if there are sex differences in myelination in the OFC or dSTR indicative of neural communication within these brain areas, protein levels of MBP and PLP in the OFC and dSTR of prepubertal and adult rats were measured in Experiment 4a. I hypothesized that control female prepubertal rats would have increased levels of MBP and PLP in the OFC but not the dSTR as compared to control males and females treated with neonatal testosterone, indicating that neurotransmission is faster in the OFC but similar in the dSTR in control females perhaps leading to increased OFC communication with and control over the dSTR in control females. I also hypothesized that a similar sex difference would be seen in adult male and female rats. Analysis of protein levels of MBP and PLP in the OFC and dSTR of prepubertal rats indicated no differences in expression levels between the sexes. However, analysis of adult rats revealed that females expressed more MBP and PLP in the OFC but similar levels in the dSTR as compared to males.

We failed to find any sex differences or effects of neonatal testosterone treatment on myelination levels in the OFC and dSTR of prepubertal rats. It is possible

that myelination within the OFC and dSTR does not contribute to the sex difference in impulsive choice behavior that was reported. It is also possible that the maturation of these brain areas is progressing at such a fast rate during this time of pubertal development that the variations from day to day and between subjects makes it very difficult to detect a difference between groups at this age. In humans, myelination of the frontal cortex continues to develop throughout puberty and is not fully developed until around 18 years of age (Gogtay et al. 2004).

Our results indicate that adult female rats have higher levels of white matter in the OFC than do adult male rats. This result is similar to the findings from an MRI study in humans that reported that women have 9 times the amount of white matter as did men in frontal and parietal brain areas (Haier et al. 2005). Increased levels of MBP and PLP are indicative of increased myelin levels, as PLP and MBP are the two most abundant proteins in CNS myelin (Boggs 2006; Greer and Lees 2002). Increased myelin levels can indicate either an increase in neuronal connections or an increase in axonal diameter of neurons. Both the number of neuronal connections and axonal diameter are associated with faster, more efficient neuronal communication (Sherman and Brophy 2005). These results indicate that the speed of neurotransmission within the OFC but not the dSTR is greater in adult female rats as compared to male rats, perhaps resulting in increased OFC communication with and control over the dSTR in females. However, it is important to note that the separate western blot assays of myelin levels in the OFC and dSTR only provide information about the levels of connectivity within these brain areas and does not provide any information about sex differences in the

direct projections from the OFC to the dSTR as these projections were severed during the dissection of each individual brain area.

To determine if there is a sex difference in the strength of projections from the OFC to the dSTR, an anterograde tracer was infused into the OFC of adult male and female rats and expression levels of the tracer in the dSTR were quantified in Experiment 4b. I hypothesized that females would have a greater expression of the tracer in the dSTR than would males, indicating that females have more projections from the OFC to the dSTR than do males. Analysis of the expression levels of the anterograde tracer in the dSTR resulted in the novel discovery that the strength of the projections from the OFC to the dSTR was greater in adult female rats as compared to adult male rats.

This finding in combination with the finding that females display increased impulsive action control as compared to males supports the hypothesis that the PFC influences impulsivity by modulating the operations of lower brain areas involved in reward-based behaviors, such as the striatum (Galvan et al. 2006; Peper et al. 2012; Perry et al. 2011). In this view, the PFC acts as a top-down modulator of the stimulus-response behaviors driven by the striatum, integrating behaviorally relevant information and preventing the over-reliance on fixed action patterns (Perry et al. 2011; Peters and Buchel 2011). These findings are in agreement with the finding that disruption of PFC output by transcranial magnetic stimulation (TMS) in humans leads to increased preference for immediate small rewards over delayed large rewards (Figner et al. 2010). The current findings also agree with the finding that lower integrity within the

frontostriatal white matter tract predicts a greater increase in impulsivity as the delay for the large reward increases (Peper et al. 2012).

It is hypothesized that the excitatory glutamatergic inputs of the OFC into the dSTR activate the indirect pathway in order to facilitate the suppression of an undesired behavior. As discussed in the Introduction, projections from the striatum diverge into two routes: the direct pathway leading to excitation of thalamic projections to motor cortex and the indirect pathway leading to inhibition of thalamic projections to motor cortex. The balance between these two pathways allows for the release of desired behavioral patterns while inhibiting undesired behavioral patterns (Miller and Buschman 2007). The increased strength of projections from the OFC to the dSTR and increased myelination within the OFC in adult female as compared to male rats may lead to increased control of the OFC over the dSTR leading to increased inhibition (via the indirect pathway) of thalamic projections to motor cortex, thereby dampening motor output and enhancing the ability of females to inhibit undesirable behaviors.

Finally, to determine if OFC communication with and control over the dSTR plays a role in the increased levels of impulse control in females as compared to males, the OFC was temporarily inactivated by injection of lidocaine into the OFC of adult male and female rats during performance of an impulsive action task in Experiment 4c. I hypothesized that if a sex difference in impulsive action is the result of females having greater OFC control over the dSTR than males, then the inactivation of the OFC should decrease female impulsive action levels to that of males, eliminating the sex difference in impulsive action. Results revealed that inactivation of the OFC failed to have an effect

on impulsive action responding in adult male and female rats. The present study had a small number of animals in each group, four males and four females. Initial plans were to add another replicate of animals to the study in order to increase statistical power. However, after noticing the apparent lack of a lidocaine-induced effect on performance, we opted not to add another replicate to the study to conserve animal use. However, in hindsight, adding another replicate to the study may have helped to determine if the lidocaine-induced inactivation of the OFC was more disruptive to the performance of males as compared to females. Lidocaine dampens signal conduction in neurons by blocking voltage-gated sodium channels in the axonal membrane that are responsible for signal propagation (Ragsdale et al. 1996). If the dose of lidocaine used in this study affected male performance more than female performance, it might suggest that females were better able to overcome the lidocaine suppression of OFC activity because they may have more projections than do males from the OFC to the dSTR to help compensate for this decrease in OFC neuronal firing.

The tendency for females to make more, albeit insignificantly, premature responses than male in the aCSF vehicle condition appears perplexing and counter to our findings in Experiment 2b. However, in order to shorten the length of training and focus on impulsive action several changes were made from the standard 5-CSRTT. For example, only one stimulus location was used, the stimulus duration was twice as long (1 sec as compared to 0.5 sec), the ITIs were lengthened during the Unpredictable ITI condition, and during training only one successful session was required before moving to the next training level as compared to two successful sessions. One study that

examined sex differences in impulsive action on a simplified version of the 5-CSRTT using only two stimulus locations and a stimulus duration of 2.5 sec reported that adult female rats made more impulsive actions than did adult male rats when the time before the presentation of the stimulus was increased (Burton and Fletcher 2012). By shorting the training period, we may have unknowingly introduced extraneous variables unrelated to impulsive action control, such as anxiety, stress, and the rate of acquisition of the task, which could have affected impulsive action control performance in our study. However, this is only speculation and the data from the small number of animals tested do not support any sex differences or effects of lidocaine-induced inactivation of the OFC on impulsive action responding in adult rats. However, further behavioral experiments with functional manipulations of the OFC and dSTR should be conducted to test the hypothesis that greater OFC control over the dSTR in females as compared to males provides females with a greater ability than males to inhibit undesirable behaviors.

### **Conclusions, implications, and future directions**

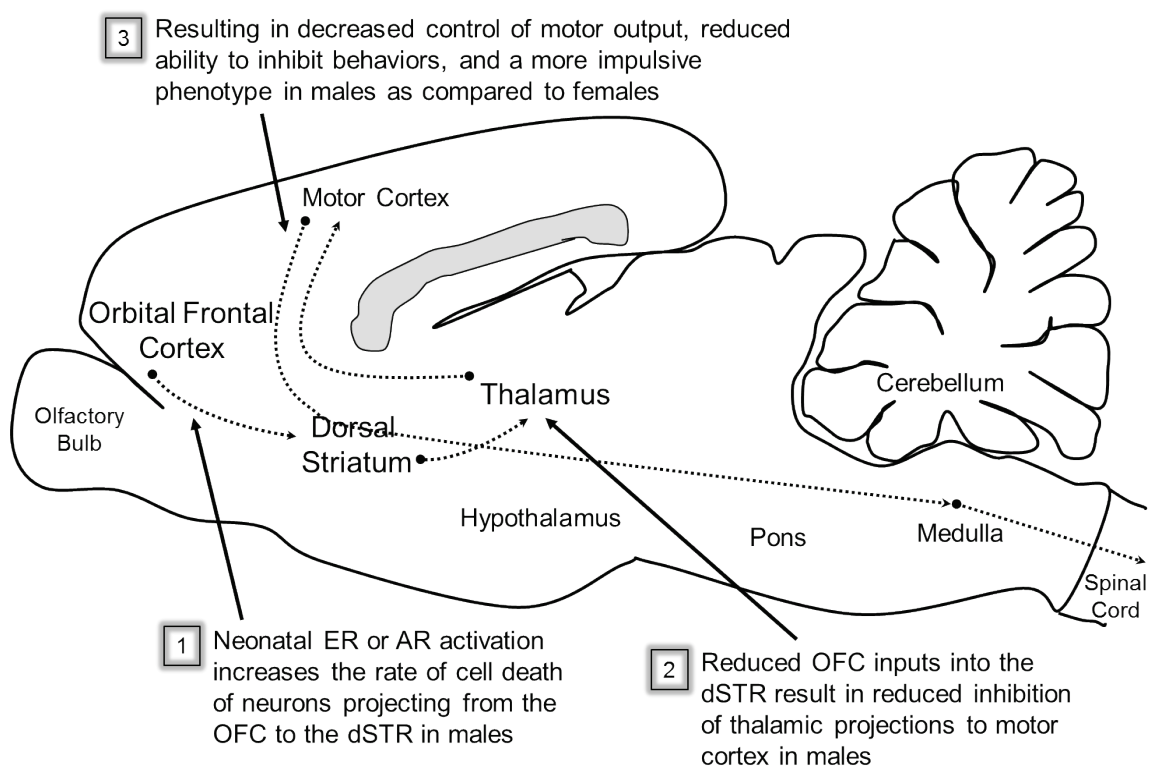
The current set of experiments demonstrate a sex difference in impulsive choice behavior in prepubertal rats that is organized neonatally by the actions of both androgens and estrogens and a sex difference in impulsive action behavior in adult rats. In addition, these experiments establish a molecular sex difference in adult rats that could possibly underlie the enhanced impulse control in females as compared to males. It is unclear if the sex differences in myelination within the OFC and strength of the

projections from the OFC to the dSTR in adult rats is organized by neonatal hormone levels. Future studies are needed to determine if neonatal estrogen or androgen receptor activation can masculinize myelination within the OFC or the projections from the OFC to the dSTR in adult animals. However, it is possible that epigenetic changes caused by neonatal activation of estrogen or androgen receptors could cause changes in the rate of apoptotic cell death during development that might produce the sex difference in the number of cells that project from the OFC to the dSTR. Sex differences in cell number could be established through neurogenesis, neuronal migration, phenotypical differentiation, or cell death. Of these neurodevelopmental events, cell death has received the majority of the support as the most common mechanism by which sex differences in cell number are established (Forger 2009). Cell death is a naturally-occurring and essential process in the brain, and over 50% of neurons born during neurodevelopment undergo apoptosis or programmed cell death during development (Oppenheim 1991).

Expression of high levels of the Bax protein promotes apoptosis, whereas expression of high levels of the Bcl-2 protein protects neurons from apoptosis (Forger 2009). Neonatal activation of estrogen or androgen receptors could affect the development of impulsive behavior by increasing HAT activity at the promoter region of the Bax gene in neurons projecting from the OFC to the dSTR during neonatal development in males resulting in increased gene transcription of the Bax protein and ensuing cell death of these neurons producing an increased impulsive phenotype later in life (Figure 36). Deletion of the Bax gene eliminates the sex difference in cell number in

the BNST (Forger et al. 2004; Gotsiridze et al. 2007), and the human Bcl-2 gene contains the sequence for the estrogen-response element (Perillo et al. 2000). So, it is plausible that neonatal activation of estrogen receptors could alter the rate of cell death in specific brain areas in a sex-dependent manner. However, future studies should be conducted to test this hypothesis.

**Figure 36**



**Figure 36.** Proposed model of molecular mechanism of the neurodevelopment of impulsive behavior in male rats. ER: estrogen receptor; AR: androgen receptor; OFC: orbital frontal cortex; dSTR: dorsal striatum

It is appropriate to note here that the decreased levels of impulse control in males as compared to females should not necessarily be view as a disadvantage. The

sexes are merely different in their average levels of impulsive responding. Any sex difference that evolves was able to form because that behavior when conducted by one member of a sex proved to be advantageous for that individual sex or for the opposite sex. Impulsive responding becomes detrimental and disadvantageous only when levels of impulsivity become excessive as is seen in impulsivity disorders, such as ADHD, which is more common in males than in females (Holden 2005; Swanson et al. 1998).

The knowledge that male rats display increased impulsive behavior as compared to female rats and that this behavior is organized by early life exposure to testosterone is interesting from a basic science standpoint but can also inform us about the development of impulsive behaviors in humans. As both humans and rodents are mammals, their endocrine and central nervous systems share many properties in common (Nelson 2005b). Their estrogen and androgen hormones and receptors are very similar (Blaustein 2008), and the process of sexual differentiation is also very similar in the two species (Nelson 2005a). Comparable to our finding in rodents, girls display increased inhibitory control as compared to boys. For example, delayed gratification scores are higher for girls than they are for boys when asked to withhold from eating candy until instructed to do so by an interviewer (Li-Grining 2007). Furthermore, girls are rated by their parents as having greater inhibitory control than are boys on the 13-item inhibitory control subscale of the Child Behavior Questionnaire (Moilanen et al. 2009). These inhibitory control ratings increase with age at similar rates in both boys and girls, but girls continue to display greater inhibitory control as compared to age-matched males. The majority of the research into sex differences in

inhibitory control has focused on pre-school aged children with few studies examining inhibitory control after puberty. However, one study in which college students were given a questionnaire that contained choices between receiving an immediate small reward or a delayed large reward found that males chose the immediate small reward more often than did females (Kirby and Marakovic 1996). In addition, disorders of impulsive behavior, such as ADHD and pathological gambling, are more frequently diagnosed in men than in women (Holden 2005; Johansson et al. 2009; Swanson et al. 1998; Tavares et al. 2001). These similarities between rodents and humans suggest that the levels of neonatal testosterone, its metabolites, and their receptors in the brain during neurodevelopment may be important for impulse control in humans as well. Changes to the prenatal hormonal environment in humans could perhaps cause long lasting epigenetic changes in the epigenome of males that result in reduced impulse control and a lower threshold to make a motor response or action.

In addition to the natural variation of neonatal hormone levels between the sexes and between individuals of the same sex, other factors can alter the amount of hormonal activity during development. For example, exogenous environmental factors, such as stress and endocrine disruption, could also contribute to individual differences in impulse control. It should be noted that genetic predisposition and natural prenatal hormonal levels most likely play the largest part in determining impulse control level. However, other exogenous factors could play a smaller role. For example, maternal stress can disrupt endocrine functioning and increase androgen levels in pregnant women (Cruess et al. 2000; Sarkar et al. 2007). Recent studies in humans have shown

that a wide variety of prenatal stressors (e.g., high anxiety, stressful personal life-events, natural disasters, perinatal complications) increase the risk for a diverse range of neurodevelopmental disorders (O'Donnell et al. 2009). Very early prenatal stress has been shown to cause deficits in social behaviors and learning in male, but not female mice (Mueller and Bale 2007; Mueller and Bale 2008), suggesting that this early prenatal time frame may be a critical time window in which environmental effects are important in the sex dependent development of impulsive behaviors.

Endocrine disrupting chemicals (EDCs) could also contribute to differences in impulse control levels. EDCs are exogenous compounds that interfere with the natural aspects of hormone action (Zoeller et al. 2012). Prenatal exposure to EDCs, such as bisphenol A (BPA) primarily used in plastics, polychlorinated biphenyls (PCBs) used as a coolant and insulating fluid, and methoxychlor (MXC) used as a pesticide, can cause changes in the volume and protein expression of sexually dimorphic brain regions and negatively affect many behaviors later in life (For review see, Gore 2008). PCBs, for example, were banned from industrial use in 1976 due to their deleterious health effects. However, because PCBs are very stable compounds and do not decompose readily these chemicals remain pervasive in the soil, water, and air and can enter the body through our diets (Steinberg et al. 2008; Sugawara et al. 2006). Levels of EDCs reported in maternal blood are usually low enough to have no deleterious effects on mothers but can have devastating effects on the much smaller fetus during extremely critical times for brain development (Crews et al. 2000). Endocrine disruption could also affect prenatal development in a sex-specific manner and contribute to the sex bias in

ADHD as males are exposed to prenatally higher levels of testosterone and its metabolites, DHT and estradiol (McCarthy 1994). It is possible that early prenatal stress and/or prenatal exposure to high levels of EDCs could disrupt estrogenic and androgenic functioning during neurodevelopment leading to decreased projections from the OFC to the dSTR resulting in increased levels of impulsivity later in life. Future studies would need to be conducted in order to test this hypothesis.

It is important to note that in the current set of experiments, behavioral and molecular sex differences were discovered in separate unrelated experiments. Future experiments are needed to determine if there is a causal relationship between alterations in the projections from the OFC to the dSTR and changes in impulsive behavior. Furthermore, our finding of increased strength of projections from the OFC to the dSTR in females as compared to males does not provide any specificity as to whether these projections synapse of striatal neurons in the direct or indirect pathway. Further studies are needed to test the hypothesis that these projections cause excitation of the indirect pathway. In addition, future studies are needed to determine if the sex difference in impulsive action seen in adults is organized by the neonatal hormonal environment.

In conclusion, the present experiments demonstrate for the first time that there is a sex difference in impulsive choice control in prepubertal rats that is organized neonatally by the actions of both androgens and estrogens, demonstrating that the organizational effects of testosterone extend to PFC-dependent behavior. This sex difference subsides in adulthood, but a sex difference in impulsive action control is

present in adulthood. The present studies indicate that there are no sex differences in the levels of the transporters for dopamine or norepinephrine in the OFC or dSTR in male and female prepubertal or adult rats. Lastly, the present studies report the novel discovery that adult female rats have increased levels of myelination within the OFC and increased strength of projections from the OFC to the dSTR as compared to adult male rats. These findings support the hypothesis that increased OFC communication with and control over the dSTR provides females with a greater ability as compared to males to inhibit undesirable behaviors. The sex differences reported here have implications for the understanding of sex differences in impulsivity in the general population and for the development of appropriate treatments for males and females with neuropsychological disorders, such as ADHD, pathological gambling, and drug addiction.

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