The effects of 17β-estradiol on striatal dopamine transmission and its implications for responsivity to antipsychotics in female rats.

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A Thesis
In the Department
of
Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy (Psychology) at
Concordia University
Montreal, Quebec, Canada

November 2017

CONCORDIA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

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and submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (Psychology)

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ABSTRACT

The effects of 17β-estradiol on striatal dopamine transmission and its implication for responsivity to antipsychotics in female rats.

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There are sex differences in the symptom manifestation of schizophrenia as well as in the response to antipsychotics. For example, women respond better to antipsychotic treatment when estrogens are used as an adjuvant therapy. This has led to the question of whether sex hormones play a role in the neurobiology of this disorder and/or the response to treatments. The earliest version of the dopamine hypothesis of schizophrenia proposed that this disorder is associated with hyperactive striatal dopamine transmission and most antipsychotics antagonize dopamine D2 receptors. Yet, estrogens themselves have been shown to enhance striatal dopamine transmission; suggesting a paradox.

This thesis examined the relationship between one estrogen, 17β-estradiol, and dopamine, and the mechanisms through which 17β-estradiol affects D2 receptors. Specifically, the effects of 17β-estradiol on tonic/phasic dorsal striatal dopamine release was measured. The effects of 17β-estradiol on specifically phasic dorsal striatal dopamine release was examined. It was shown that 17β-estradiol rapidly increases both tonic and phasic dorsal striatal dopamine release *in vivo* in anesthetized female rats.

The rodent model of amphetamine sensitization, which mimics in rats some of the neuronal changes thought to occur in schizophrenia, was then used for the ensuing two studies. In those experiments, the mechanisms through which 17β-estradiol and the antipsychotic, haloperidol, affect dopamine transmission were explored via their effects on dopamine D2 receptor affinity and some of its second messenger proteins (i.e., Akt, phosphorylated Akt, and β-arrestin). Both 17β-estradiol and haloperidol increase the affinity of $D2^{High}$ and $D2^{Low}$ receptors. Additionally, the combination of 17β-estradiol and haloperidol together decrease the proportion of dopamine D2 receptors in the high affinity state. However, there were no observed effects on second messenger proteins.

These studies provide one potential mechanism, i.e., via dopamine D2 receptor affinity state, through which 17β-estradiol in conjunction with the antipsychotic, haloperidol, may enhance its effects on reducing striatal dopamine transmission despite increasing dopamine release. These data have implications for the beneficial effects of estrogens in the treatment of schizophrenia in women.

ACKNOWLEDGMENTS

How do you discuss over seven years of gratitude in a single document? I don't think it's a question I can answer. However, one of the many things that this journey has taught me is to never give up! So, here's my attempt at trying to answer my own question, because I know I wouldn't be the person I am today, having accomplished all that I have, without these people in my life, for whom I am forever grateful.

I would like to first and foremost acknowledge and extend my gratitude to my supervisor, Dr. Wayne G. Brake. He has been an invaluable leader and has taught me a great deal about science and conducting research through this journey. His support, guidance, patience, and encouragement throughout my research career at CSBN have been priceless. Thank you for helping me learn how to write like a scientist. Me fail English? Unpossible!

I would like to thank my internal committee members Dr. Peter Shizgal and Dr. Jim Pfaus, for their critical and insightful feedback, which helped me improve my thesis. A special thank you to Dr. Paul Joyce and Dr. Lalit Srivastava for kindly agreeing to be a part of my thesis examining committee. I would also like to thank Dr. Alison Fleming, without whom Chapter 4 would have been impossible to complete on time.

I would not have been able to complete any experiments without the amazing support of the CSBN staff. I am very grateful to Heshmat Rajabi for his countless hours spent helping me troubleshoot the HPLC, and helping me conduct all my studies on time by finding ways to get every piece of equipment I ever needed, by any means necessary. Thank you to Isabelle Bouvier for all of her behind the scene leg work and support, and to Aileen Murray and her ACF staff for their day-to-day hard work in keeping the animals healthy and well maintained. The CSBN staff's assistance throughout this journey is greatly appreciated and one that will never be forgotten.

I would also like to extend my gratitude to all of my Honors Thesis and volunteer students who spent immeasurable hours helping me with my experiments and tolerating me with all my craziness. In particular, I would like to thank Christian Sanio, Fei Xiang Zhou, Jesse Duchemin,

Collin Gagne, Ashley Reynolds, and Piriyah Thirunavukkarasoe for their dedication to data collection, I could not have done it without them.

I was very fortunate to find a home-away-from-home in Montreal, and more importantly, a second family within the CSBN. This family was with me through thick and thin, and became my wings, inspiring me along the way. Lindsay, Firas, Marie-Pierre, Dean, Tracey, Mayte, Emily, Sherri, and Milan, all really helped me learn how to crawl, walk, and run as a graduate student. They started as my office mates and peers, but in a very short period of time turned into my partners in crime in academia. Whether it was the trips to Costco through Vendome, France, eating lunch together in the grad lounge, sharing a walk together for a coffee break, having dinner across the hall (Friends style!), practicing presentation late nights, or surviving the HPLC room, COMPs, voltammetry, and all that grad school has to offer, they all have been supportive and helpful through every step of the process. Thanks to each member of this family, I missed my home in Mississauga a little less, I outlasted the roller coaster of a ride that is grad school, but most importantly, thanks to this family, my time in Montreal was irreplaceable and invaluable. I could not have survived grad school without all of their support, thank you, thank you, thank you!

I would like to thank all of my fellow lab members, both past and present. In particular, Dr. Anne Almey, Dr. Dan Madularu, Dr. Dema Hussain, Dr. Nicole Gervais, and Czarina Evangelista – they tolerated my insanity, respected my threatening letters regarding the well-being of my microdialysis probes or work space, but most importantly, they were also my partners in crime for enduring all the obstacles the research gods threw our way!

I also want to thank all of my Sauga friends, in particular, Ravi, Sherry, and Trikki, for their support and patience. I promise I was doing school work whenever I wasn't hanging out with you guys!

I want to acknowledge and extend my upmost gratitude to my amazing parents Shams and Tasneem, my in-laws Ghazanfar and Nadeema, my siblings Soaleha, Nida, Ghayas, Shahid, Usman, Abeer, Nauman, and Faizan, and my niece and nephew, Sophia and Zaviar, for their

unconditional love and support. I am who I am because of them and I can say with great pride and joy that I would not have gotten this far if it were not for their love and support. I dedicate this degree to you Mom and Dad!

Madiha, your love, encouragement, and infinite support is the reason why I have made it this far. You are the most important person in my life, my source of strength, hope, wisdom, and inspiration. You have helped me stay grounded throughout grad school, helped me keep a sense of humor when I lost my own, and helped me keep my sanity when insanity seemed like the only option. I couldn't have done it without you and it wouldn't have been worth doing without you. Thank you and I love you!

Throughout my studies I have received funding from FQRNT, Concordia Merit Scholarship, CSBN/FRSQ Entry Stipend, and Faculty of Arts and Science Graduate Fellowship. I would like to acknowledge their support and thank FQRNT, the CSBN, and the Faculty of Arts and Science for their generosity.

CONTRIBUTION OF AUTHORS

Chapter 2: These experiments were conceived by Waqqas Shams in conjunction with Wayne Brake based on previous research in this lab by Matthew Quinlan and Wayne Brake. Waqqas Shams conducted these experiments under the supervision of Wayne Brake in collaboration with an undergraduate thesis student: Christian Sanio.

Chapter 3: This study was conceived by Waqqas Shams, Marie-Pierre Cossette and Wayne Brake. Waqqas Shams conducted this experiment under the co-supervision of Peter Shizgal and Wayne Brake, while Marie-Pierre Cossette provided the methodological expertise throughout the experiment.

Chapter 4: These experiments were conceived by Waqqas Shams in conjunction with Wayne Brake with methodological expertise provided by Marc Morissette and Thérèse Di Paolo. Waqqas Shams and Marc Morissette conducted these experiments under the co-supervision of Thérèse Di Paolo and Wayne Brake in collaboration with Anne Almey and Alison Fleming.

Chapter 5: These studies were conceived by Waqqas Shams in conjunction with Wayne Brake. Waqqas Shams conducted these experiments under the supervision of Wayne Brake in collaboration with undergraduate thesis students: Fei Xiang Zhou, Jesse Duchemin, and Collin Gagne.

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LIST OF ABBREVIATIONS

AMPH……Amphetamine
ANOVA……Analysis of variance
AP……Anteroposterior
ATP……..Adenosine triphosphate
cAMP……...Cyclic adenosine monophosphate
CYC……..Cyclodextrin
D1R……...Dopamine D1 receptor
D2R……...Dopamine D2 receptor
DA……Dopamine
DAT……Dopamine transporter
DOPAC……...3,4-dihydroxyphenylacetic acid
DS……..Dorsal striatum
DSM……..Diagnostic and statistical manual of mental disorders
DV……Dorsoventral
E2……..17β-estradiol
ER……..Estrogen receptor
FSCV……..Fast-scan cyclic voltammetry
GABA……-aminobutyric acid
GPCRs……...G-protein coupled receptors
GPER-1……G-protein coupled estrogen receptor-1
GPR 30……..G-protein coupled receptor 30
GRK……..G-protein receptor kinase
HAL……Haloperidol
HPLC……...High performance liquid chromatography
HVA……Homovanillic acid
MAM……Methylazoxymethanol
mER……Membrane-associated estrogen receptor
ML……...Medial-lateral
mPFC……..Medial prefrontal cortex
NA……..Nucleus accumbens

NMDA……*N*-methyl-D-aspartate

OVX……Ovariectomized

pAkt……Phosphorylated Akt

PE……..Polyethylene

PFC……Prefrontal cortex

PP2A……Protein phosphatase 2A

SERM……Selective estrogen receptor modulator

SN……Substantia nigra

VS……Ventral striatum

VTA……...Ventral tegmental area

CHAPTER 1:
GENERAL INTRODUCTION

Schizophrenia

Schizophrenia is a chronic neurodevelopmental and debilitating disorder. In 1919, Emil Kraeplin, a German psychiatrist, named this disorder as *dementia praecox* referring to the chronic form of the illness. A Swiss psychiatrist named Eugen Bleuler chose the term *schizophrenia*, which means "split brain," to refer to the dissociation between the affective and cognitive features of individuals with this disorder (*for review, see* Charley and Nestler, 2004). Since then, the definition of the term *schizophrenia* has evolved further, as can be seen in the different editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM). Schizophrenia is currently characterized by psychotic symptoms including hallucinations, delusions, flattened affect, difficulty with goal directed behavior, impairment in memory, and/or executive functioning (American Psychiatric Association, 2000). Although disturbances in motor abilities and cognition can be observed earlier in life, the clinical symptoms of this disorder typically appear in late adolescence or early adulthood.

Schizophrenia is a life-long disorder that affects individuals at every level of socioeconomic status. Although schizophrenia is considered as a single disease, recent findings suggest that it may be comprised of a group of disorders with different etiologies (American Psychiatric Association, 2000; Sadock and Sadock, 2007). Schizophrenia is the most common of the psychotic disorders, which include schizoaffective disorder, substance-induced psychotic disorders, major depressive disorder with psychotic features and psychotic disorders due to general medication condition (Seeman, 2007). It affects roughly 1% of the population worldwide, although incidence may differ across subpopulations (Konopaske and Lewis, 2007).

There are a number of risk factors for schizophrenia. Family, twin, and adoption studies suggest that there are genetic risk factors for schizophrenia (Kendler and Diehl, 1993; van Os and Kapur, 2009). Up to 108 loci have been implicated in the development of schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). There are also non-genetic risk factors that include infection during fetal development (Brown, 2006; Brown and Susser, 2008), anoxia at birth, trauma during childhood, brain injury, isolation, and brain lesions (*for review, see* Seeman, 2011). In addition, steroid and psychostimulant drug abuse, and psychosocial stress are also risk factors (van Os and Kapur, 2009; *for review, see* Seeman, 2011). It is important to note that no single risk factor causes schizophrenia. In fact, it is believed that both genetic and non-genetic risk factors probably combine to produce some level of

vulnerability to the disorder. This is called the stress-vulnerability model (Zubin and Spring, 1977).

The symptoms of schizophrenia can surface as early as the teenage years, and those affected with the disorder can meet the criteria for diagnosis in late teenage years or in their early twenties (Seeman, 2007). In men the onset of symptoms is typically between the ages of 15 and 25, whereas in women the onset can be between the ages of 20 and 30 (Sham et al., 1994). In contrast to men, women display a bimodal age of onset distribution with a second peak of onset occurring around menopause (Sham et al., 1994). It should be noted that this finding is controversial with others arguing that there is no second peak in women (Baldwin and Srivastava, 2015). It is commonly believed that schizophrenia is more common in men than women (Beauchamp and Gagnon, 2004; McGrath, 2006; *for review, see* Salem and Kring, 1998). Although more men with the disorder are hospitalized than women (Beauchamp and Gagnon, 2004), this can be accounted for by the fact that men develop schizophrenia at an earlier age than women. This is thought to be because estrogens have been shown to serve as a protective agent against schizophrenia (*for review, see* Markham, 2012). Nonetheless, although schizophrenia affects men and women differently, it affects them in equal numbers (Seeman, 1997).

Estrogens and schizophrenia

Estrogens are a class of steroid hormones that include estrone, estriol, and estradiol. Estrone, primarily secreted during menopause, and estriol, primarily secreted during pregnancy, are metabolites of estradiol (Lazari et al., 2009; Litwack, 2010). Estradiol is lipophilic and able to pass through the blood-brain-barrier and cell membranes (Litwack, 2010). It is the most potent and most abundant of the estrogens in female mammals during their reproductive years (*for review, see* Luine, 2014). Estrogens are synthesized in the gonads, and to a lesser extent, in the brain. They are ovarian hormones important for female reproduction (Behl, 2001) and influencing development of the brain (Kölsch and Rao, 2002).

Estrogens induce their effects by binding to estrogen receptors (ERs). There are three currently known ERs; ERα, ERβ, and G-protein coupled estrogen receptor 1 (GPER-1). GPER-1 is the same receptor previously known by its former orphan receptor name, GPR-30. ERα and ERβ are classical nuclear receptors. Historically, these receptors have been found in the cytoplasm. From there they translocate to the nucleus where estrogens bind to them. Specifically,

activation of these nuclear ERs (located in nuclei and in the cytoplasm) leads to translocation to nuclear DNA, leading to changes in gene transcription and ultimately altering protein synthesis (Litwack, 2010). GPER-1 as the name implies, is imbedded within the cell membrane and putatively interacts with G-proteins. Activation of GPER-1 potentially activates multiple second messenger cascades and has been shown to increase intracellular Ca^{2+} (*for review, see* Almey et al., 2015).

Recently, ERα and ERβ, which were thought to be exclusively nuclear receptors (which normally reside in the cytoplasm until migrating into the nucleus) were found at the cell membrane in certain areas of the brain. In such areas of the brain, they are referred to as membrane-associated estrogen receptors (mERs). They are not embedded within the cell membrane *per se*, rather are found as either homo or hetero-dimers adjacent to the cell membrane (Boonyaratanakornkit and Edwards, 2007). Activation of membrane-associated ERα or ERβ can induce similar intracellular events as G-protein coupled receptors. Specifically, activation of mERs triggers protein kinase cascades, which lead to phosphorylation of transcription factors and thus gene transcription (Blaustein, 2010). ERα and ERβ are both nuclear and membrane-associated receptors, while GPER-1 is only a membrane receptor.

ERα, ERβ, and GPER-1 mRNA are abundantly expressed throughout the central nervous system although the relative distribution of the mRNA of these receptors differs. For example, ERα mRNA is observed in the cerebral cortex, ERβ mRNA is observed in the ventral striatum (VS) and the hippocampus, while GPER-1 mRNA is observed in the VS and the hippocampus (Shughrue et al., 1997). Additionally, electron microscopy revealed that there is ERα, ERβ, and GPER-1 protein immunoreactivity in the dorsal striatum (DS; Almey et al., 2012) and the medial prefrontal cortex (mPFC; Montague et al., 2008; Almey et al., 2014), and ERα immunoreactivity has been found in the hippocampus as well (Montague et al., 2008). Research by Almey and colleagues (Almey et al., 2012, 2014) demonstrated that these receptors are localized outside the nucleus at the pre- and post-synaptic membranes.

Estrogens have been shown to exert protective effects in individuals with schizophrenia (Bergemann and Riecher-Rössler, 2005). For example, Kraepelin in 1909 and Kretschmer in 1921 reported that women suffering from schizophrenia display physical signs and anatomical abnormalities due to reduced functionality of the ovaries (*for review, see* Bergemann and Riecher-Rössler, 2005). They believed this was caused by hypoestrogenism (estrogen

deficiency). Riecher-Rössler and Häfner (1993) found lower serum estrogen levels in women with schizophrenia. These women also reported having irregular cycles (*for review, see* Riecher-Rössler and Häfner, 1993). For example, Krafft-Ebing in 1896 reported that women displayed psychotic behavior before or during menstruation (Bergemann and Riecher-Rössler, 2005), and that the late onset of schizophrenia in women is caused by the loss of ovarian function (Riecher-Rössler and Häfner, 1993).

Estrogens have been shown to delay the onset of schizophrenia (Häfner et al., 1991; Lindamer et al., 1997; Häfner et al., 1998; Bergemann et al., 2002; Bergemann and Riecher-Rössler, 2005). For example, Cohen and colleagues (1999) found that early puberty in women is associated with the onset of schizophrenia at a later age. In fact, the onset of schizophrenia in women is typically seen during the two phases of life when estrogen fluctuation is at its greatest viz. puberty and menopause (Prior, 1998). Seeman and Lang (1990) found that the onset of schizophrenia occurs more frequently in women during times when their estrogen levels are low. Additionally, lower levels of circulating estrogens have been reported in women who develop schizophrenia (*for review, see* Markham, 2012). Symptoms become more severe during and before menstruation, when estrogen levels are low (Seeman and Lang, 1990). Cases have been reported where the severity of symptoms can be improved by oral contraceptive treatment alone (Felthous et al., 1980; Tunde-Ayinmode et al., 2008), and this is also seen in postmenopausal women (Lindamer et al., 1997).

Women require lower doses of antipsychotics between the ages of 20 – 40 years, during a time of high ovarian estrogen production, in comparison to older women or men of the same age (Seeman, 1983). Alternatively, following menopause, when ovarian estrogen production is at its lowest, women require higher doses of antipsychotics in comparison to men of the same age (Seeman, 1983; Riecher-Rössler and Häfner, 1993). In fact, several studies found that estrogen levels have an inverse correlation to dose of antipsychotics, such that as estrogen levels increase, women with schizophrenia require lower antipsychotic doses and vice versa (Hallonquist et al., 1993; Seeman, 1994; Gattaz et al., 1994; Riecher-Rössler et al., 1994). Women receiving estrogen therapy in conjunction with antipsychotics have a better response to treatment than those treated with antipsychotics alone (Kulkarni et al., 1996, 2001; Akhondzadeh et al., 2003). Furthermore, women with schizophrenia display significant improvement in severity of symptoms during pregnancy, a time when estrogen levels are highest (Chang and Renshaw,

1986; Kendell et al., 1987; Lindamer et al., 1997). However, post-partum, when estrogen levels decrease dramatically, symptom severity is enhanced (Nott, 1982; Kendell et al., 1987; Lindamer et al., 1997).

More recently, selective estrogen receptor modulators (SERMs) have been used as an adjunctive treatment along with antipsychotics. For example, raloxifene, a first-generation SERM, has been effective along with antipsychotics in improving symptoms in women with schizophrenia (Usall et al., 2011; *for review, see* Weickert et al., 2016). Specifically, 60 mg of raloxifene administered daily for 12 weeks along with antipsychotic medication improved positive and negative symptoms (Usall et al., 2011), and executive function and memory (Huerta-Ramos et al., 2015) in postmenopausal women with schizophrenia. In addition, when antipsychotic treatment was combined with 120 mg of raloxifene administered daily for six weeks, attention and memory improved in middle-aged women with schizophrenia (Weickert et al., 2015). These findings provide some evidence that gonadal hormones like estrogens, and SERMs, modulate the severity of schizophrenia, and the effects of antipsychotics.

Symptoms of schizophrenia

Schizophrenia includes three subtypes of symptoms; positive, negative, and cognitive. Positive symptoms include an excess or distortion of normal functions (American Psychiatric Association, 2000). They reflect an increase in the presence of abnormal behaviors. These symptoms include delusions, hallucinations, speech disturbances, disorganized behavior, thought irregularity, and movement irregularity (Konopaske and Lewis, 2007). Negative symptoms include restrictions in the range and intensity of behaviors (American Psychiatric Association, 2000). They reflect an absence of behaviors normally observed in healthy individuals. These symptoms include reduction in emotional expression (affective flattening), reduction in fluency or productivity of thought and speech (alogia), restriction in initiation of goal-directed behavior (avolition), absence of ability to experience pleasure (anhedonia), and attentional impairment (Konopaske and Lewis, 2007). Cognitive symptoms, which can be the most debilitating, may impair people from carrying out everyday tasks. They are deficits in or inability to organize one's life and to work sequentially and effectively (Simpson et al., 2010). Cognitive symptoms include impairment in language and learning (Konopaske and Lewis, 2007), deficits in semantic and explicit memory, attention, working memory, and executive function (Simpson et al., 2010).

There are a number of neurotransmitters implicated in these symptoms, such as dopamine (DA), glutamate, serotonin, acetylcholine, and γ-aminobutyric acid (GABA). A great deal of research has focused on DA. While the exact neuropathology of schizophrenia has not been fully elucidated, most evidence suggests DA transmission plays a major role. The earliest version of the DA hypothesis of schizophrenia (*for review, see* Baumeister and Francis, 2002) proposes that this disorder is caused by hyperactive DA transmission (Seeman, 1987). It is thought that DA plays a major role in schizophrenia largely because most of the successful treatments of this disorder collectively act on DA receptors in one way or another. In fact, most antipsychotics have in common the ability to block the DA D2 receptor (D2R).

DA neurons implicated in schizophrenia

DA transmission is important for cognition, motor control, movement, learning, motivation, and reward (*for review, see* Bannon et al., 2012). The time course of DA release can best be represented by a continuum (Schultz, 2000), ranging from sustained "tonic" to episodic "phasic" release (Grace, 1991). Historically, the terms tonic and phasic were used to describe extrasynaptic versus synaptic DA release (Grace, 1991) and primarily addressed the discrepancy between the techniques used for DA detection, viz. microdialysis, electrophysiology, or voltammetry (*for review, see* Grace, 2016). Since then, the definitions have been modified to address DA neuronal activity states. For example, tonic DA release refers to slow, steady-state, irregular firing via extra-synaptic DA release, while phasic DA release refers to rapid, high-amplitude, burst firing via intra-synaptic DA release (Hollerman et al., 1992; Moore et al., 1998; Floresco et al., 2013; Grace, 2016). These two types of release also correspond with different behavioral responses. For example, tonic DA release has been shown to be involved in motivation (Cagniard et al., 2006; Salamone and Correa, 2012), effort (Salamone et al., 2009), and response vigor (Niv et al., 2007; Guitart-Masip et al., 2011), while phasic DA release has been implicated in reward expectation (Mikhailova et al., 2016), learning, motivated approach, and effort-based decision making (Hamid et al., 2016).

Tonic and phasic DA release are influenced by neurons within the basal ganglia. For example, GABAergic projections from the ventral pallidum to ventral tegmental area (VTA) and substantia nigra (SN) DA neurons (Grace and Bunney, 1984) keep a subset of these DA neurons in a silent, non-firing, hyperpolarized state (Grace, 2016). On the other hand, projections from

the pedunculopontine tegmentum act on *N*-methyl-D-aspartate (NMDA) receptors on DA neurons to generate phasic burst firing. As such, pedunculopontine tegmentum projections regulate phasic DA release, whereas the ventral pallidum, through its actions on a subset of DA neurons, regulates tonic DA release. In a similar manner, the ventral pallidum can also determine the level of amplification of the phasic DA release (Grace, 2016).

Tonic DA release is dependent upon spontaneous baseline spike activity and stimulation of NMDA receptors. NMDA receptors on DA neuron terminals are stimulated by cortical glutamatergic projections, resulting in an increase of intracellular calcium and vesicular DA release. Tonic DA release represents a low level of extracellular DA regulated by cortical afferents (Grace, 1991). Phasic DA release refers to burst spike activity relying on glutamatergic excitation of DAergic neurons (Floresco et al., 2003). Produced by burst firing of DA neurons, phasic DA release occurs in response to behaviorally relevant stimuli, and is rapidly terminated by immediate re-uptake into presynaptic terminals via DA transporters (Grace, 1991; Floresco et al., 2003).

There are two major DA neuron pathways in the brain *viz.* the mesocorticolimbic DA pathway and the nigrostriatal DA pathway (Ungerstedt, 1971; *for review, see* Neve, 2010). The mesocorticolimbic DA pathway consists of cell bodies located in the VTA that predominantly project terminals in the VS and the prefrontal cortex (PFC). The nigrostriatal DA pathway consists of cell bodies located in the SN that predominantly project to terminal fields in the DS. Both of these pathways are implicated in schizophrenia (Neve, 2010). Some of the brain regions thought to be involved in schizophrenia pathology are the VS, the DS, the mPFC, and the hippocampus (*for review, see* Meyer-Lindenberg, 2010). DA is robustly released within these brain regions in the basal ganglia including the VS, the DS, the PFC, and the SN (*for review, see* Bannon et al., 2012).

DA receptors

Up until the 1980s, it was thought that there were two DA receptors, DA D1 receptors (D1Rs) and D2Rs (Kebabian and Calne, 1979). These two receptors are different in their pharmacological, physiological, and biological properties, their anatomical distribution, and their effects on the second messenger cascade systems (Kebabian and Calne, 1979). In the 1990s, three new DA receptors were identified, D3, D4, and D5 (*for review, see* Civelli and Borrelli,

2010). However, based on the sequences and pharmacological properties, these receptors could be classified into two families, D1-like and D2-like receptor families (Civelli et al., 1993). The D1-like receptor family is comprised of the D1 and D5 DA receptors. They are highly homologous and are quite variable in their level of affinity, although they both share similar affinities to D1R antagonist drugs such as SCH23390 (Civelli and Borrelli, 2010). Most drugs that act on D1Rs also act on DA D5 receptors. The D2-like receptor family is comprised of the D2, D3, and D4 DA receptors. This family of DA receptors has roughly 40% homology, and vary little in the level of affinity of ligands. Most drugs that act on D2Rs also act on DA D3 and D4 receptors. Both the D1-like and D2-like receptor families are found on the post-synaptic membrane. However, only D2-like receptors are found on the pre-synaptic membrane. And only the D2 and D3 receptors can act as autoreceptors when located on the presynaptic membrane (Mansour and Watson Jr., 2000). All DA receptors are seven transmembrane receptors coupled to guanine nucleotide-binding regulatory proteins (G-proteins), and thus are referred to as G-protein coupled receptors (GPCRs; *for review, see* Civelli and Borrelli, 2010).

GPCRs are activated through binding of a ligand or a neurotransmitter. Upon binding, just like any other receptor, the GPCRs undergo a conformational change. When activated, GPCRs activate a second messenger cascade system within the cell. One of the purposes of a second messenger cascade system is signal amplification. GPCRs are metabotropic receptors requiring milliseconds for a response to occur (Ford et al., 2009).

D2Rs and antipsychotic treatment

Searching for a common target for the action of all antipsychotic medications at the time, Philip Seeman discovered a common binding site in 1975 (Seeman et al., 1975, 1976). He initially called the common binding site of antipsychotics the DA antipsychotic receptor (Seeman et al., 1975, 1976), and it was later discovered to be the D2R (Kebabian and Calne, 1979). In fact, it was found that the clinical efficacy of antipsychotics was directly correlated with D2R occupancy, around 60 – 75% (Farde et al., 1988; *for review, see* Seeman, 2006). At roughly 80% D2R occupancy by either typical or atypical antipsychotics, extrapyramidal side effects can occur (Seeman, 2002, 2004).

More recently, Seeman discovered that individuals with schizophrenia have a higher level of D2Rs in the high affinity state, in comparison to individuals without schizophrenia or those

with other forms of psychoses (*for review, see* Seeman 2006, 2010, 2011). D2Rs have two affinity states: high affinity or low affinity (Wreggett and Seeman, 1984). D2Rs in the high affinity state are active (George et al., 1985), and in the low affinity state are inactive (Seeman, 1982). When in the high affinity state, a ligand or a neurotransmitter binds to the D2Rs and this in turn triggers the G_i protein to inhibit the adenylyl cyclase, preventing it from converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP; McDonald et al., 1984; George et al., 1985; *for review, see* Neve, 2010). When D2Rs are in the low affinity state, DA does not readily bind to them (McDonald et al., 1984; George et al., 1985).

There is a greater density of high affinity D2Rs present in the brains of unmedicated individuals with schizophrenia than there are in individuals without schizophrenia or other forms of psychoses (Seeman, 1987). In an animal study, the affinity state of D2Rs in the striatum of adult male rats was measured. Male rats chronically treated with the atypical antipsychotic, olanzapine, exhibited a 2.4 fold increase in binding at these receptors (*for review, see* Seeman et al., 2005).

Shvartsburd et al. (1983) found that individuals with schizophrenia chronically treated with haloperidol (HAL) displayed constant high steady-state plasma levels of HAL. Such a finding is troublesome. Sustained high steady-state plasma levels of HAL have been shown to increase D2R binding (Samaha et al., 2008). Additionally, sustained HAL levels convert a higher percentage of D2Rs into the high affinity state (Samaha et al., 2008). As such, if an individual with schizophrenia discontinues their treatment, the increase in binding to D2R can be detrimental. This is important because individuals with schizophrenia undergoing treatment with antipsychotic drugs have a high noncompliance rate. The rate of noncompliance is on average 50% (Agarwal et al., 1998; Naber and Karow, 2001), but has been shown to be as high as 89% (Dolder et al., 2002). Individuals taking antipsychotics often discontinue treatment as soon as they notice improvements in their symptoms (e.g. fewer hallucinations or delusions). This is due to the adverse side effects of the treatment. Discontinuing antipsychotic treatment results in worsening of the symptoms. In fact, stopping treatment can worsen the symptoms by two fold (Seeman, 2011). Discontinuation of treatment can result in an increase in the density of high affinity D2Rs on the post-synaptic membrane, a phenomenon referred to as *DA supersensitivity* (Muller and Seeman, 1978; *for review, see* Seeman, 2006, 2010, 2011).

DA supersensitivity

Chronic antipsychotic treatment can produce a state of DA supersensitivity. It is not detrimental or damaging as long as the treatment is not discontinued. However, once an individual discontinues treatment, the increase in high affinity D2Rs present on the post-synaptic membrane can be detrimental. Antipsychotic treatment-induced DA supersensitivity can worsen psychotic symptoms upon discontinuation of treatment (Muller and Seeman, 1978; *for review, see* Seeman, 2006, 2010, 2011). Up to 40% of individuals administered chronic antipsychotic treatment develop this phenomenon. Lieberman and colleagues (1987) reviewed the theory of DA supersensitivity and the effects it has on individuals with schizophrenia with or without antipsychotic treatment. They found that these individuals are behaviorally supersensitive to psychostimulant drugs (e.g. amphetamine (AMPH) or methylphenidate) that increase DA release. Roughly 75% of those without treatment experience worsening of symptoms after taking such psychostimulant drugs (Lieberman et al., 1987; Curran et al., 2004). Additionally, roughly 40% of antipsychotic-treated individuals with schizophrenia experience worsening of symptoms after a single administration of psychostimulant as since psychostimulant drugs increase DA release, the increased psychotic action of these drugs could stem from supersensitivity of postsynaptic D2Rs (*for review, see* Seeman, 2011). To better understand this phenomenon, Samaha and colleagues (2007) investigated whether it can be observed during ongoing antipsychotic treatment, in adult male rats. They found that with chronic treatment, both HAL and olanzapine lose their effects, leading to treatment failure, due to the development of DA supersensitivity (Samaha et al., 2007). Thus, these data show that chronic antipsychotic treatment is not an advantageous form of pharmacotherapy, since it can produce a state of DA supersensitivity, making the treatment ineffective.

Location and function of D1 and D2 receptors in brain regions associated with schizophrenia

DA receptors are mostly present within the basal ganglia and both the mesocorticolimbic and the nigrostriatal DA pathways (*for review, see* Neve, 2010). Specifically, the VS, the DS, the PFC, and the hippocampus contain DA receptors. Unlike the VS and DS, both of which contain the most D2Rs (Boyson et al., 1986), the majority of DA receptors in the PFC are D1Rs (Boyson et al., 1986; Lidow et al., 1989; Lidow and Goldman-Rakic, 1994; Liggins, 2009). The

hippocampus also contains both D1Rs and D2Rs, but to a lesser extent (*for review, see* Beaulieu and Gainetdinov, 2011).

Under normal conditions DA can bind to either the D1-like or the D2-like receptor family. In the case of the D1-like receptors, once DA is bound, the G_s protein activates adenylyl cyclase. The activated adenylyl cyclase transforms ATP into cAMP, a second messenger (Neve et al., 2004; Bockaert, 2009). Alternatively, D2-like receptors typically couple to G_i proteins. One role of G_i proteins is to inhibit the enzymatic activity of adenylyl cyclase. The G subunit of the G_i protein inhibits adenylyl cyclase, preventing it from transforming ATP into cAMP. This net result, in turn, prevents signal amplification of this particular second messenger cascade (Neve et al., 2004; Bockaert, 2009; *see* Figure 1).

In addition, D2Rs can activate other second messenger cascades. For example, ligand binding to some D2Rs results in the phosphorylation of G-protein receptor kinase (GRK). This phosphorylation/activation of GRK in turn activates β-arrestin, a scaffolding protein critical for GPCR desensitization and trafficking (Beaulieu et al., 2007). Such effects influence other second messenger cascades including those that recruit β-arrestins and result in a complex formation between β-arrestin, protein phosphatase 2A (PP2A; a protein that is important for regulation of the cell cycle, signal transduction, cell differentiation, and transformation), and protein kinase B, also referred to as Akt. The formation of this complex results in dephosphorylation/deactivation of Akt (Beaulieu et al., 2007, *see* Figure 1)). Akt is a kinase that is important for a number of functions including inhibiting cell death (apoptosis), which in turn promotes cell survival and proliferation.

Lower levels of Akt have been observed in the post-mortem brain tissue of individuals with schizophrenia (Emamian et al., 2004). Some antipsychotics have been shown to phosphorylate Akt (Masri et al., 2008). Interestingly, estrogen has been shown to rapidly stimulate phosphorylation of Akt in the hippocampus, both mice and rats (McEwen et al., 2001; Akama and McEwen, 2003; Znamensky et al., 2003; Yildirim et al., 2011). In addition, 17β-estradiol (E2) has been shown to enhance DA release (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Xiao and Becker, 1994; Bazzett and Becker, 1994; Becker and Rudick, 1999) and affect D2Rs (Di Paolo et al., 1988; Levesque and Di Paolo, 1988). Thus, it is reasonable to postulate that estrogen may be interacting with antipsychotic medications via this mechanism.

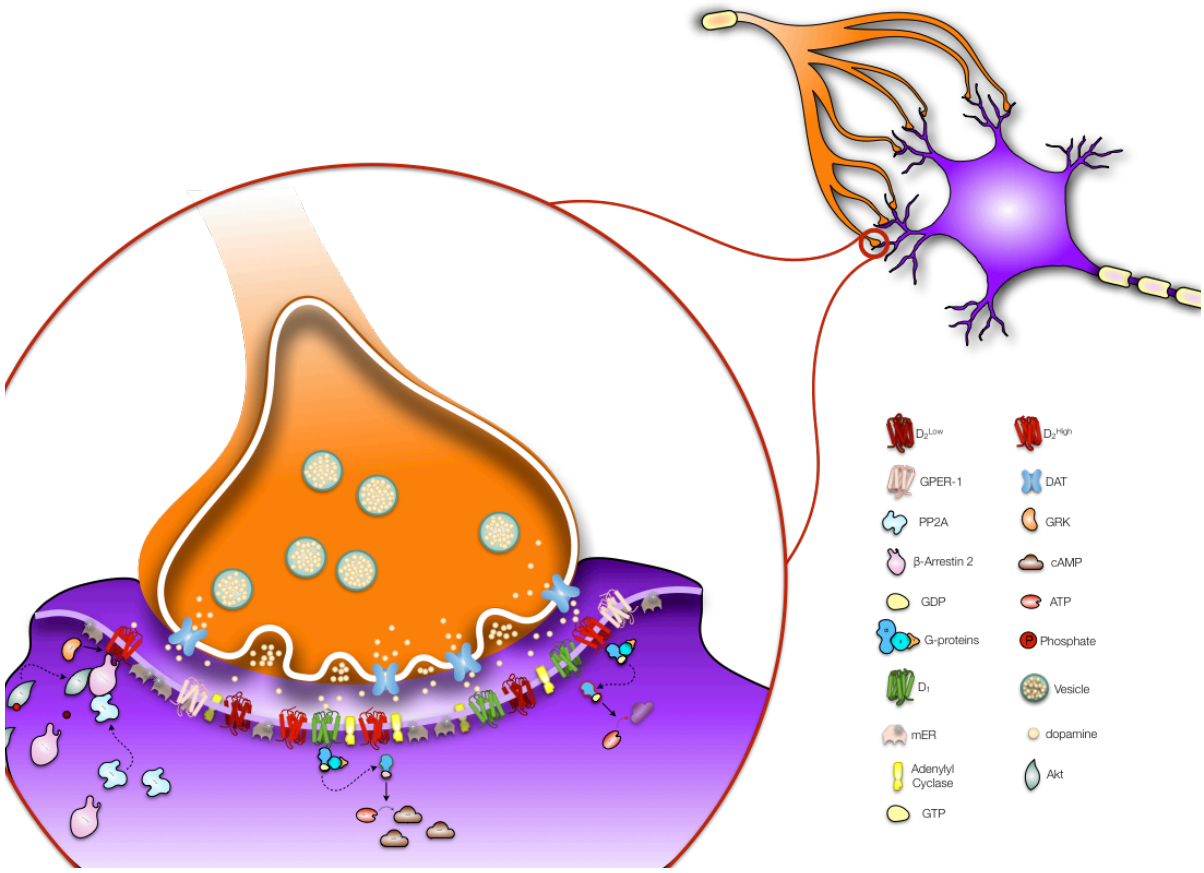

Figure 1. Conceptualization of a striatal synapse illustrating activation of DA D1 and D2 receptors. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; PP2A = protein phosphatase 2A; GRK = G-protein receptor kinase; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; ATP = adenosine triphosphate; mER = membrane-associated estrogen receptor; GTP = guanosine triphosphate.

Animal model of AMPH-sensitization

Much of what we understand about schizophrenia comes from animal studies, and to a lesser extent, experimental human studies. These studies help to reproduce some of the relevant symptomatology of schizophrenia, its treatment, and the validity and efficacy of the treatment. Interestingly, most of the currently approved antipsychotics were validated using animal models (*for review, see* Geyer et al., 2012).

There are at least 20 different animal models mimicking the symptoms of schizophrenia, although a number of them may overlap in their methodology (Jones et al., 2011). These animal models can be divided into four different categories: neurodevelopmental, lesion, genetic, and pharmacological. Neurodevelopmental models utilize manipulations of the environment, or exposure to drugs during the sensitive perinatal period, causing irreversible changes in the development of the central nervous system (*for review, see* Jones et al., 2011). An example of a neurodevelopmental model would be gestational methylazoxymethanol (MAM), where during a critical gestational period, disruption of neurogenesis is induced in pregnant female rats (Cattabeni and Di Luca, 1997). Lesion models consist of damaging a specific neonatal brain structure causing abnormalities during development of the brain (e.g. neonatal ventral hippocampal lesion). Genetic models target silencing of a particular subset of genes (e.g. knock-out mice). Lastly, pharmacological models utilize drug administration resulting in alterations in behavior and neurobiological function. This introduction will review one pharmacological model, AMPH-sensitization.

Long-term use of AMPHs or other psychostimulants can induce psychosis in humans (Lieberman et al., 1987; Curran et al., 2004; *for review, see* Featherstone et al., 2007). Repeated administration of AMPH produces long-lasting molecular and neurochemical changes (Robinson et al., 1985). It can last many years and can be treated by antipsychotics. Repeated administration of AMPH can also produce long-lasting behavioral and neurochemical changes in animals, and is commonly referred to as behavioral sensitization (Magos, 1969; Echols, 1977; Robinson et al., 1985). It is speculated that this behavioral sensitization in animals mimics AMPH psychosis observed in humans (*for review, see* Robinson et al., 1985). Since AMPH psychosis is indistinguishable from the positive symptoms of schizophrenia (*for review, see* Featherstone et al., 2007), researchers were interested in examining the effects of repeated AMPH administration

in animals as a model of some of the symptoms and behavioral and neurochemical changes thought to occur in schizophrenia.

AMPH-sensitization can be divided into three phases: induction, withdrawal, and expression. In the induction phase a rat is given repeated exposure to AMPH. The repeated exposure to AMPH (induction) increases sensitivity to the drug, resulting in increased neurochemical and behavioral responses to AMPH (Cador et al., 1995; Vezina, 1996; Madularu et al., 2014) and other psychostimulant drugs (*for review, see* Featherstone et al., 2007). The withdrawal phase consists of terminating repeated exposure to AMPH for a duration of time. Sensitization to AMPH is a time-dependent process, such that motor effects develop one week after cessation (withdrawal; e.g. Madularu et al., 2014) and can last up to twelve months (Paulson et al., 1991; Paulson and Robinson, 1995). The expression phase consists of administering an AMPH challenge, resulting in the rat displaying enhanced locomotor behavior compared to when it was first exposed to the drug (Robinson et al., 1982; Cador et al., 1995; Samaha et al., 2007, 2008; Madularu et al., 2014; *for review, see* Robinson and Becker, 1986).

Snyder (1973) was the first to report that AMPH psychosis could be used as an experimental model for schizophrenia. Since then, a plethora of studies have used AMPH sensitization as an animal model of some of the neurobiological changes thought to occur in schizophrenia (i.e. Magos, 1969; Echols, 1977; Mamelak et al., 1985; Robinson et al., 1985; Cador et al., 1995; Vezina, 1996;). This is because AMPH sensitization can cause dysregulation of DA functions similar to those thought to occur in schizophrenia.

Repeated exposure to AMPH can induce a number of cellular, molecular, behavioral, and neurochemical changes, including changes in the mesocorticolimbic DA pathway (Kolb et al., 2003; Castner et al., 2005; Fletcher et al., 2005; *for review, see* Featherstone et al., 2007). For example, AMPH sensitization enhances locomotor activity, nucleus accumbens (NA; Samaha et al., 2007, 2008; Madularu et al., 2014) and striatal DA release, and stereotypy (Robinson and Becker, 1986; Paulson and Robinson, 1995, 1996) and all these effects can be attenuated via antipsychotic treatment (Samaha et al., 2007, 2008; Madularu et al., 2014). Specifically, we (Madularu et al., 2014) found that chronic HAL treatment in conjunction with high levels of E2, reduces AMPH-induced locomotor activity in AMPH-sensitized female rats.

Rationale and hypotheses

Most would agree, schizophrenia, partially if not primarily, involves dysregulation of DA transmission. Additionally, the onset of schizophrenia in women typically occurs around two time periods, puberty and menopause; both of which reflect drastic fluctuations in estrogens. Finally, given that the severity of its symptoms vary across the menstrual cycle, this thesis addresses the effects of estrogens (specifically E2) on DA transmission. Additionally, since the current treatment of schizophrenia primarily targets the D2R, this thesis addresses the effects of E2 on the D2R. Specifically, the effects of E2 on dorsal striatal AMPH-induced DA release, and how E2 may be having its effects on dorsal striatal D2R were examined.

In Experiment 1, the effects of dorsal striatal AMPH-induced DA release in response to E2 infusion into the DS, mPFC, or the SN, *in vivo*, via microdialysis, in anaesthetized female rats were measured. In Experiment 2, the effects of E2 on electrically-evoked dorsal striatal DA transients via fast-scan cyclic voltammetry in anaesthetized female rats were measured. The findings from Experiments 1 and 2 help further clarify the effects of E2 on DA transmission. According to the original DA hypothesis of schizophrenia, this disorder is caused by excessive DA transmission. This begs the question *how can E2 help provide a better treatment for schizophrenia if E2 increases DA transmission?* We previously found that high E2 in conjunction with the antipsychotic HAL, reduces DA release in AMPH-sensitized female rats, in comparison to non-AMPH-sensitized female rats (Madularu et al., 2014). As such, it is important to understand how E2 might be having its effects, specifically on D2Rs. Estrogen in conjunction with antipsychotics results in a greater response to treatment (Kulkarni et al., 1996, 2001; Akhondzadeh et al., 2003), and it affects the same receptor (*viz* D2R) that is bound by antipsychotics. Thus, Experiments 3 and 4 address the effects of E2 on dorsal striatal D2R. In Experiment 3, the effect of E2 on dorsal striatal D2R affinity in AMPH-sensitized female rats treated with either an antipsychotic (HAL) or saline was measured. Lastly, in Experiment 4, immunoreactivity was measured for D2R-related second messenger proteins. These include Akt, phosphorylated Akt, and β-arrestin. Levels of these second messengers were measured in AMPH-sensitized female rats with no, low, or high levels of E2, treated with HAL or saline. Experiments 1 and 2 help further clarify the effects of E2 on AMPH-induced dorsal striatal DA release, while Experiments 3 and 4 help address how E2 may be having its effects on dorsal striatal D2R.

CHAPTER 2:
17β-ESTRADIOL INFUSIONS INTO THE DORSAL STRIATUM RAPIDLY INCREASE DORSAL STRIATAL DOPAMINE RELEASE *IN VIVO*

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Citation: Neuroscience (2016); 330.

Abstract:

Systemic injections of E2 in ovariectomized female rats rapidly enhance dorsal striatal DA release in response to AMPH. Additionally, a single injection of E2 rapidly (within 30 min) enhances AMPH-induced DA release. *In situ* studies show that this rapid effect of E2 occurs specifically within the DS. The present study investigated the *in vivo* effects of E2 infused into the DS, mPFC or the SN on dorsal striatal DA release. Rats were ovariectomized and implanted with a silastic tube containing 5% E2 in cholesterol, previously shown to mimic low physiological serum concentrations of 18-32 pg/ml. Single probe microdialysis was used to measure extracellular DA levels in the DS. In addition, DA release was measured subsequent to systemic injections of the indirect DA agonist, AMPH (0.5 mg/kg SC), administered simultaneously with E2 (0.544 μg/100 μl) or its vehicle, cyclodextrin (0.520 μg/100 μl). Local infusions of E2 into the DS resulted in a greater AMPH-induced dorsal striatal DA release in comparison to vehicle. Local infusions of E2 into the mPFC or the SN did not result in an enhancement of AMPH-induced DA levels in the DS. These studies suggest that increases in dorsal striatal DA release in response to systemic E2 are a consequence of E2 actions within the DS itself.

1. Introduction

Estrogens have been shown to modulate behaviors subserved by striatal DA and its increasingly evident that they play a role in disorders that involve dysregulated striatal DA such as schizophrenia and Parkinson's (*for review, see* Seeman, 2000, 2006; Smith and Dahodwala, 2014). Previous literature provides evidence that specifically E2 modulates DA function in the DS (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999) and acts directly on striatal medium spiny neurons (Mermelstein et al., 1996). The effects of E2 on DA transmission also alter behavior in rats, while ovariectomy in rats attenuates AMPH-induced behavioral responses and DS DA release. Systemic E2 replacement in ovariectomized (OVX) rats restores and enhances AMPH-induced behavioral responses and *in vitro* DS DA release (Becker and Beer, 1986). Research conducted by Becker and Rudick (1999) has shown that systemic injections of E2 in OVX rats rapidly enhance dorsal striatal DA release in response to AMPH. Becker found that a single injection of E2 rapidly (within 30 min) enhances AMPH-induced DA release (1990a, 1990b; Castner et al., 1993). Recently, our lab has also shown that high E2 replacement in OVX rats enhances AMPH-induced DA release within the DS (Hussain et al., 2016). Additionally, it has been shown that E2-induced increases in DA release is specific to the dorsal and not VS (Cummings et al., 2014). However, this rapid increase was only shown in *in situ* or systemic injection studies.

Studies have found ERα, ERβ, and G-protein coupled receptor 30 (GPR 30; now known as GPER-1) mRNA throughout the central nervous system of the rat brain. For example, ERα mRNA was detected in the cerebral cortex (Montague et al., 2008), ERβ mRNA was detected in the VS, PFC (Shughrue et al., 1997), and SN (Quesada et al., 2007; Yamaguchi-Shima and Yuri, 2007, 2011), while GPER-1 mRNA was found in the hypothalamus, caudate nucleus (Brailoiu et al., 2007), and the SN (Quesada et al., 2007; Yamaguchi-Shima and Yuri, 2007, 2011). A recent ultrastructural study in our lab demonstrated the presence of ERα, ERβ, and GPER-1 in the DS (Almey et al., 2012) and the mPFC (Almey et al., 2014) of the female rat. These receptors are localized outside the nucleus at membrane-bound pre- and post-synaptic sites. Given the behavioral effects of E2 and that its receptors are located in the DS, mPFC, and SN, it is not

surprising that E2 has been shown to affect DA transmission in the DS in the mesocorticolimbic pathway.

Based on previous research by Becker and colleagues (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999), it was hypothesized that E2 acts directly within the DS to rapidly increase DA transmission in that region. Dorsal striatal DA release is dependent upon activity at DAergic cell bodies in the SN, and the mPFC has been shown to regulate activity of these neurons. Additionally, all of these brain regions contain ERs. Thus, E2 may be acting in these other brain regions to also modulate dorsal striatal DA release. This study also examined the effects of E2 in the mPFC and the SN on dorsal striatal AMPH-induced DA release. It was hypothesized that E2 may additionally act either in the mPFC or the SN to further enhance dorsal striatal AMPH-induced DA release. E2 or the vehicle, cyclodextrin (CYC), was infused either into the DS, mPFC, or SN, while the dialysate samples were always measured from the DS *in vivo*. Such findings will help refine our understanding of how and where in the brain E2 may be modulating striatal DA transmission to affect neuropsychiatric disorders.

2. Methods

2.1 Animals

Female Sprague Dawley rats (Charles River Laboratories, St. Constant, QC, Canada) weighing 250-350g were pair housed at 21°C in polypropylene shoebox cages with beta-chip bedding. A 12:12h reverse light/dark cycle was used with lights off at 9:00 AM. Rats had access to *ad libitum* Purina rat chow and water. All handling, testing and surgical procedures were performed during the dark phase of the diurnal cycle. All protocols were previously approved by Concordia University's animal research ethics committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Surgical Procedures, Hormone and Drug Administration

All rats were anesthetized using Isoflurane (4% for induction; 2% for maintenance; Inhalation Anaesthetic, Richmond Hill, ON, Canada) and bilaterally OVX through a dorsal incision (1cm). Post-ovariectomy, all rats were implanted with a silastic tube (1 cm long; 1.96

mm OD, 1.47 mm ID) containing 5% E2 (Sigma Chemical Co., St. Louis, MO, USA) in cholesterol (Sigma) in the nape region. For post-surgical care, all rats were administered the anti-inflammatory drug, Anafen (0.1 mL/rat, SC; MERIAL Canada Inc., Morgan Baie d'Urfe, QC, Canada), the antibiotic Penicillin G (0.2 mL/rat, intramuscular; CDMV, St. Hyacinthe, QC, Canada) and antibiotic ointment (By Pharmaceuticals Inc., Brampton, ON, Canada) was applied to the incision. The silastic tube containing E2 was previously shown to mimic low physiological serum concentrations of 18-32 pg/ml consistent with estrus (Quinlan et al., 2008; Almey et al., 2013). These implants have been shown to produce consistent E2 serum levels up to 4 weeks following implant (Almey et al., 2013). Post-surgery, all of the rats were individually housed and allowed at least one week to recover in their home cages. Post recovery, the rats were pseudorandomly assigned to one of the three experimental brain areas of interest based on the experiment. The rats were tested between days 8 and 21 post ovariectomy.

Eighteen rats were pseudorandomly assigned to either the E2 (n = 9) or CYC (n = 9) group in Experiment 1. One-week post-ovariectomy surgery, each rat was once again anaesthetized using Isoflurane (4%) and placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA) and was maintained under anesthesia (2%) throughout the experiment. A three-pronged cannula (21 Ga stainless steel, 40 mm in length, 0.5 mm apart in a parallel manner) was implanted into the right or left DS with the center cannula aimed at the following coordinates from bregma: anteroposterior (AP), -0.3 mm, medial-lateral (ML), ± 3.5 mm and dorsoventral (DV), -3.0 mm (Paxinos and Watson, 2007). The three-pronged outer cannulae were used to hold microinjectors for the intracerebral infusions, while the center cannula was used to house the probe (*see* Figure 1).

Fourteen rats across two groups were used (E2; n = 7; CYC; n = 7) in Experiment 2. Preparation of the rats were similar to the description in Experiment 1, except that in Experiment 2 a two-pronged cannula (21 Ga stainless steel, 40 mm in length, 1.2 mm apart in a parallel manner) was implanted into the mPFC at the following coordinates from bregma: AP: 2.8 mm, ML ± 0.6 mm, and DV: -4.0 mm (Paxinos and Watson, 2007). The two-pronged cannulae were used to hold microinjectors for the intracerebral infusions (*see* Figure 1). A single-pronged cannula (21 Ga stainless steel, 40 mm in length), used to house the probe, was implanted into the

right or left DS with following coordinates from bregma: AP: -0.3 mm, ML ± 3.5 mm, and DV: -3.0 mm (Paxinos and Watson, 2007).

In Experiment 3, eighteen rats were used between the two groups, E2 (n = 9) and CYC (n = 9). Preparation of the rats were similar to descriptions in Experiments 1 and 2, except that in Experiment 3 a two-pronged cannula (21 Ga stainless steel, 38 mm in length, 4.8 mm apart in a parallel manner) was implanted into the SN bilaterally at the following coordinates from bregma: AP: -4.8 mm, ML ± 2.4 mm, and DV: -7.0 mm (Paxinos and Watson, 2007). The two-pronged cannulae were used to hold microinjectors for the intracerebral infusions (*see* Figure 1). A single-pronged cannula (21 Ga stainless steel, 40 mm in length), used to house the probe, was implanted into the right or left DS with following coordinates from bregma: AP: -0.3 mm, ML ± 3.5 mm, and DV: -3.0 mm (Paxinos and Watson, 2007).

In the DS, E2 or CYC were always infused in either right or left side, and dialysate samples were collected from that same side. In the mPFC and SN on the other hand, E2 or CYC were infused into both sides and dialysate samples were collected from either right or left DS.

2.3 Microdialysis

Probes were assembled according to previously reported methods (Sorge et al., 2005). They consisted of 20-µm diameter polyethylene (PE) tubing (30-35 cm long; Plastics-One, Roanoke, VA, USA) with one end connected to an infusion pump (KD Scientific; Holliston, MA, USA). Dialysate was collected from the outlet of the probe (fused silica tubing) into 0.5 ml Eppendorf tubes (Sigma-Aldrich). The other end of the PE tubing was attached to a probe tip consisting of 26 Ga stainless steel tubing, 40 mm in length (Fisher Scientific, Nepean, ON, Canada) and a 4 mm long semi-permeable membrane (280 µm OD, 220 µm ID; with a molecular weight cut off of 13 000 Da; Fisher Scientific, Nepean, ON, Canada). The outer end membrane was occluded with epoxy syringe glue (Henkel, Mississauga, ON, Canada) to create a closed system for the flow of dialysate. One end of the small-diameter fused silica tubing (110 µm OD, 41 µm ID; Polymicro Technologies; Phoenix, AZ, USA) extended into the probe 0.5 mm from the glued tip of the semi-permeable membrane. The other end of the fused silica tubing extended out of the probe, serving as an outlet for the probe.

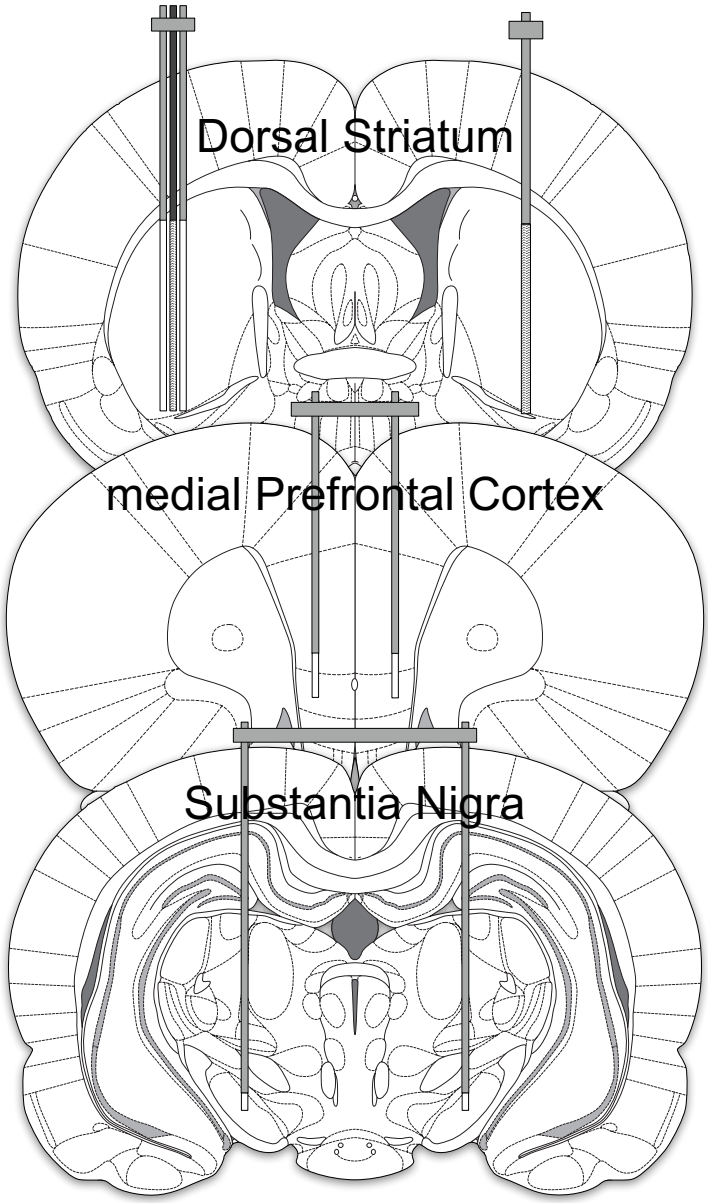

Figure 1. Target sites of microdialysis probes and injectors in the DS, mPFC, and SN.

Prior to the collection of dialysate samples for microdialysis, probes were lowered into the cannula 2 hours before dialysate sampling began. When lowered, the probe extended 4 mm beyond the guide cannula directing the probe tip and membrane towards the center of the DS. Artificial cerebrospinal fluid (145 mM Na^+, 2.7 mM K^+, 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}, 150 mM Cl^-, 0.2 mM ascorbate, 2mM Na_2HPO_4, pH 7.4 ± 0.1; Sigma) was perfused through the probe, at a rate of 1.0 µl/min, during a period of 2 hours to prevent occlusion and stabilize the baseline.

During the experiment all rats received a local (1.0 µl) intracerebral microinfusion of either E2-CYC complex (244.8 pg/µl; Sigma), or the vehicle, CYC (5.196 ng/µl; Sigma), at a rate of 1.0 µl/min for a total duration of one minute. E2 was encapsulated in CYC to make it water-soluble. E2-CYC and CYC infusion concentrations used in the current study are twice as high as that used for intracerebral microinjections in which E2 infusions had an enhancing effect on memory behavior maze task (Packard and Teather, 1997; Zurkovsky et al., 2007). We have shown this dose of E2 to be effective when infused into the mPFC to alter memory system bias (Almey et al., 2014). All of the rats were also administered an injection of AMPH (0.5 mg/kg IP) dissolved in 0.9% saline.

2.4 Procedure

Two hours after probe insertion, six baseline dialysate samples were collected. Each sample was collected for 10 min at a flow rate of 1.0 µl/min resulting in 10 µl of dialysate/sample. Samples were immediately placed in dry ice and stored at -80 °C. After baseline, each rat was administered an intracerebral microinfusion of either E2 or CYC at a rate of 1.0 µl/min for a total volume of 1.0 µl during one minute and the injectors remained in place for an additional minute to allow diffusion of the drug. Microinjectors were made from 26 Ga stainless steel tubes connected to 10 µl Hamilton syringes via PE tubing. The Hamilton syringes were attached to a variable speed electric syringe infusion pump (Harvard Apparatus, South Natick, MA, USA). The tips of the microinjectors extended 1mm beyond the end of the two or three-pronged cannulae. All microinfusions occurred at the start of the 10-minute sampling bin. During the microinfusion, rats were also administered AMPH (0.5 mg/kg IP). Following the drug administration another 12 samples were collected. Upon completion of microdialysis, methylene blue was infused at a rate of 1.0 µl/min into the guide cannulae to later verify the placements. The rats were immediately decapitated following the infusion of the dye. The brains were

removed and flash frozen using 2-methylbutane (Isopentane) in dry ice and stored at -80 °C until being coronally sectioned at 40 μm for confirmation of guide cannulae placements.

2.5 High performance liquid chromatography (HPLC)

Dialysate samples were thawed and immediately analyzed for DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), using HPLC with electrochemical detection (for detailed methods *see* Madularu et al., 2014). The samples were loaded through manual injection ports (Reodyn 7125; 20 μl loop) onto C-18 reverse-phase columns (5 μm, 15 cm; Higgins Analytical, Mountain View, CA, USA). DA and its metabolites (DOPAC and HVA) were measured on separate independent channels with dual-channel ESA coulometric detectors (Coulochem III, with a 5011 model analytical cell) for reduction and/or oxidation currents. Mobile phase was circulated through at a flow rate of 1.1 ml/min by Waters 515 HPLC pumps (Waters, QC, Canada), and consisted of 20% acetonitrile 40ml, 0.076 M sodium dodecyl sulphate, 0.1 M EDTA, 0.058 M $NaPO_4$, 0.27 M citric acid with a pH of 3.35. Known amounts of standard DA and its metabolites concentrations ([DA] 1.536 pg/μl; [DOPAC] 2.7 ng/μl; [HVA] 2.7 ng/μl; Sigma-Aldrich) were used as standards using estimates from peak heights by comparison with standard injections. Extracellular levels of DA (elution time ~ 6.5 min) and its metabolites (DOPAC elution time ~ 2.25 min; HVA elution time ~ 3.7 min) were analyzed using the EZChrom Chromatography Software Data system (Scientific Software, San Ramon, CA). The data presented are expressed as percentage baseline. To calculate percent baseline, the last 3 baseline samples prior to any drug manipulation were averaged and each sample value in picograms per microliter (pg/μl) was then divided by the average to express the data as change in DA release.

2.6 Statistical Analyses

The effects of hormone or vehicle across time were analyzed using a two-way mixed analysis of variance with time as a within-factor and hormone group as a between-factor. Analysis of variances (ANOVAs) were conducted separately for the baseline DA response and the response following drug and hormone infusion. Data are also represented as each individual rat receiving E2 in comparison to the mean response to CYC. All data were tested for and confirmed to have normal distributions and equal variances.

3. Results

3.1 Experiment 1

In Experiment 1, E2 or CYC was infused directly into the DS while measuring AMPH-induced DA release from the DS. Data from one rat were removed due to incorrect cannula implantation; thus a final *N* of 17 was used for the analyses (*see* Figure 2A). Baseline values of those rats assigned to either E2 or CYC conditions were not statistically significantly different (*see* Table 1 for absolute baseline values). No significant differences in DOPAC or HVA levels were observed between E2 and CYC (Figure 6A and 6D). DA release was increased in response to AMPH, as reflected by a significant main effect of time ($F_{(11,165)} = 13.078$, $p < 0.001$; $\eta_p^2 = .466$). AMPH-induced DA release was significantly higher in the E2 group ($F_{(1,15)} = 4.924$, $p = 0.042$; $\eta_p^2 = .247$), in comparison to the CYC group, when E2 or CYC were directly infused into the DS (*see* Figure 3A). The results of this experiment are consistent with the findings by Becker and colleagues, such that administration of E2 causes an increase in AMPH-induced DA release in the DS (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999).

3.2 Experiment 2

In Experiment 2, E2 or CYC were infused directly into the mPFC while measuring AMPH-induced DA release from the DS. Baseline values of those rats assigned to either E2 or CYC conditions were not statistically significantly different (*see* Table 1 for absolute baseline values). No significant differences in DOPAC or HVA levels were observed between E2 and CYC (Figure 6B and 6E). AMPH increased DA release as reflected by a significant main effect of time ($F_{(11,132)} = 19.193$, $p < 0.001$; $\eta_p^2 = .615$). AMPH-induced DA release did not differ between the two treatment groups ($F_{(1,12)} = 0.198$, $p = 0.664$; $\eta_p^2 = .016$; *see* Figure 4A). The findings of this study suggest that rats infused with E2 into the mPFC did not display a change in AMPH-induced DA levels in comparison to their controls.

3.3 Experiment 3

In Experiment 3, E2 or CYC was infused directly into the SN while measuring AMPH-induced DA release from the DS. Data from one rat were removed due to attrition, thus, a final *N* of 17 was used for the analyses (*see* Figures 2D and 2E). Baseline values of those rats assigned to either E2 or CYC conditions were not statistically significantly different (*see* Table 1 for absolute baseline values). No significant differences in DOPAC or HVA levels were observed

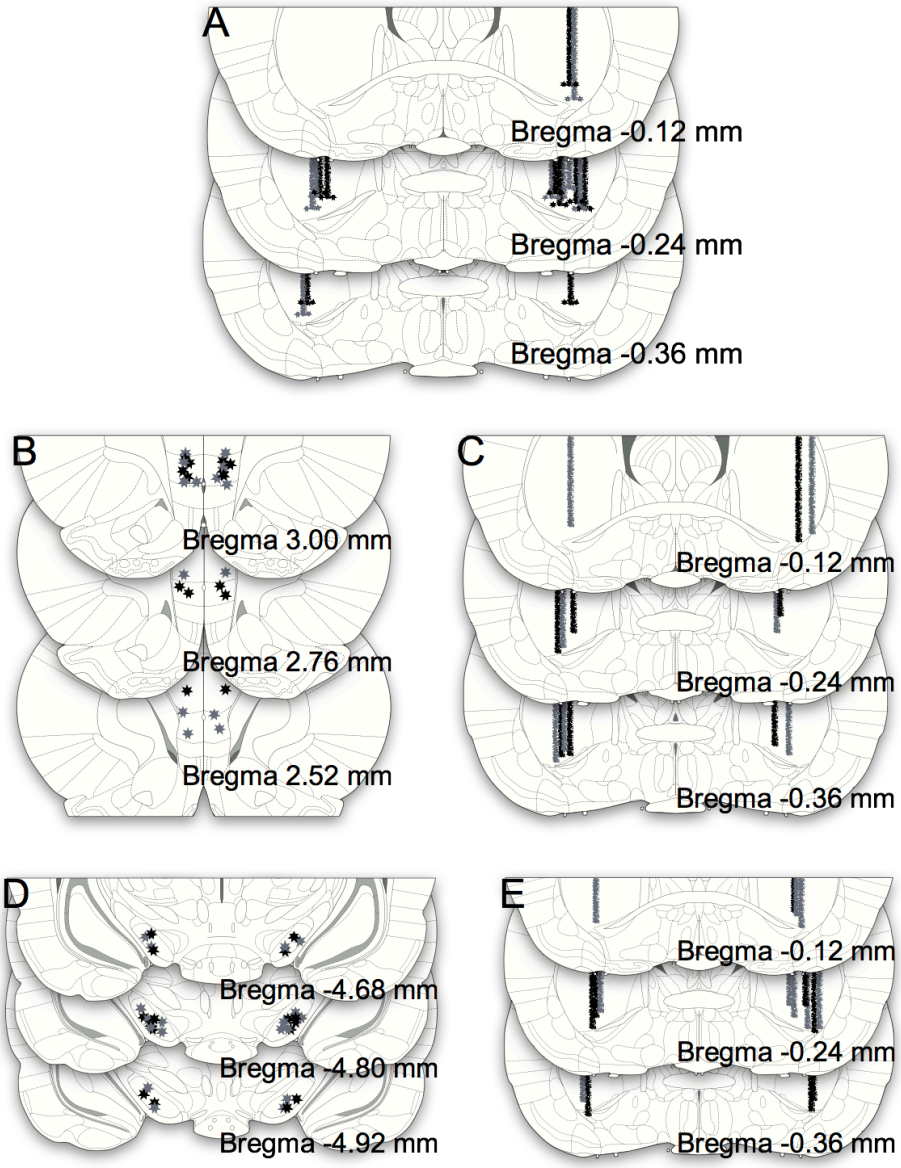

Figure 2. Schematic representation of **A**) probe and injector placements in the DS in Experiment 1; **B**) injector placements in the mPFC in Experiment 2; **C**) probe placements in the DS in Experiment 2; **D**) injector placements in the SN in Experiment 3; **E**) probe placements in the DS in Experiment 3. Black lines represent all E2 group probe placements, while gray lines represent all CYC group probe placements. Black stars represent all E2 group cannula placements for injectors, while gray stars represent all CYC group cannula placements for injectors.

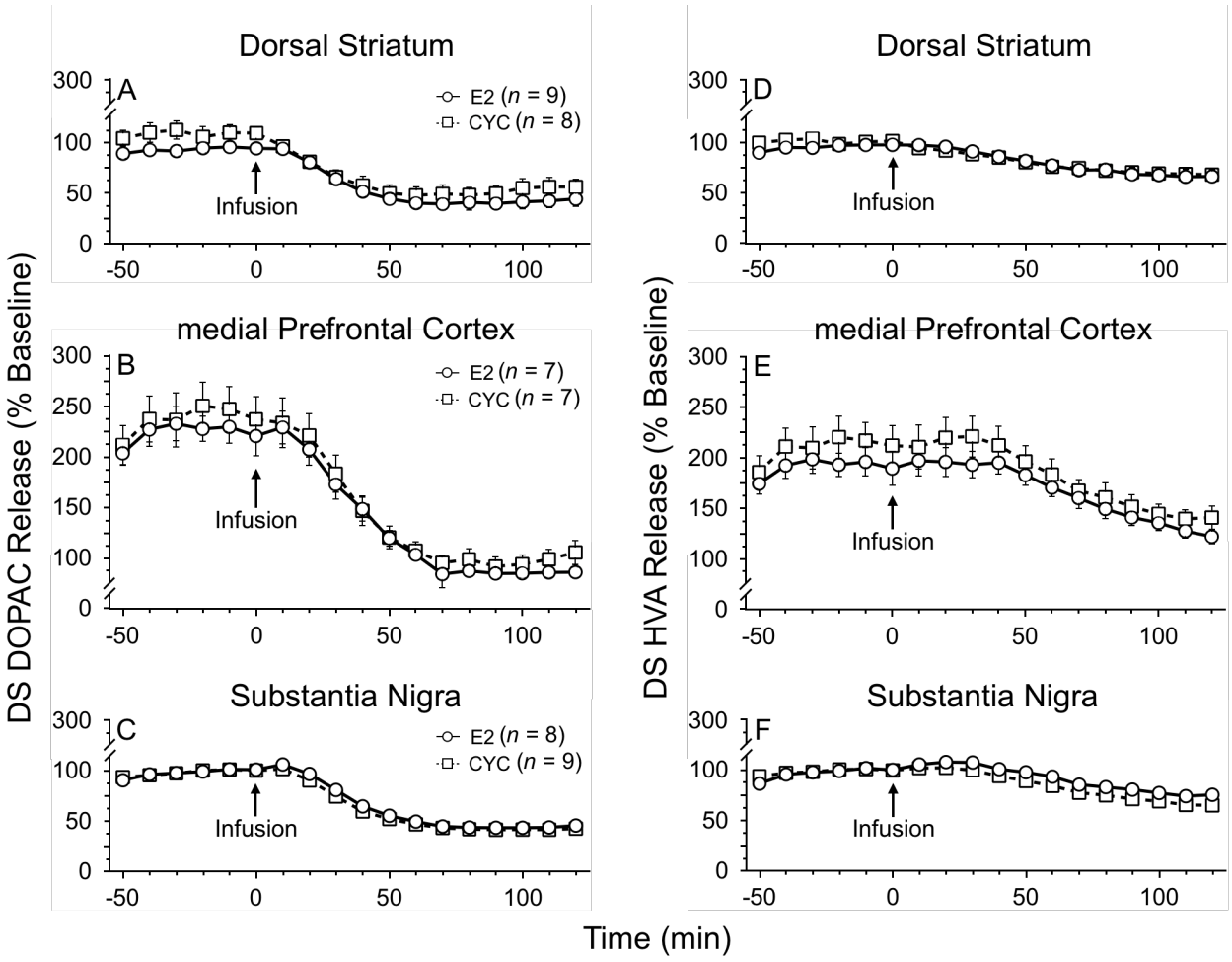

Figure 6. DOPAC or HVA in dialysate from DS after E2 or CYC infusion expressed as percentage baseline. Arrow indicates time at which AMPH was administered IP. Dorsal striatal DOPAC release in response to E2/CYC infusion in the **A**) DS; **B**) mPFC; or **C**) SN. Dorsal striatal HVA release in response to E2/CYC infusion in the **D**) DS; **E**) mPFC; or **F**) SN. DS = dorsal striatum; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; E2 = 17β-estradiol; CYC = cyclodextrin; AMPH = amphetamine.

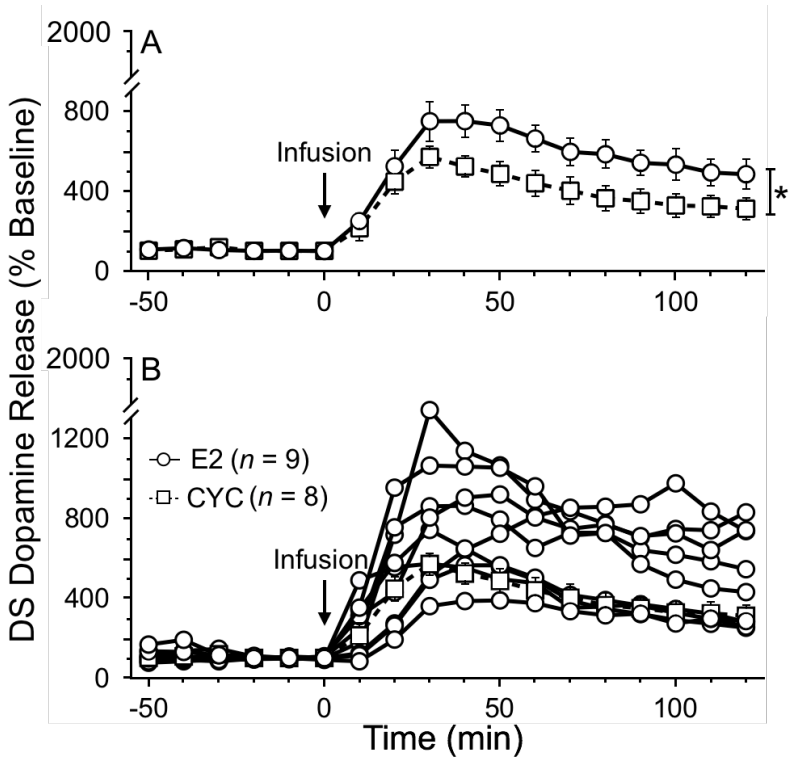

Figure 3. DA in dialysate from DS after E2 or CYC infusion into the DS expressed as percentage baseline. Arrow indicates time at which AMPH was administered IP. **A**) Group data; **B**) each individual rat in the E2 group. Error bars represent standard error of the mean. DS = dorsal striatum; E2 = 17β-estradiol; CYC = cyclodextrin; AMPH = amphetamine. * = significant main effect of drug ($p < .042$).

Table 1. Absolute baseline and peak dorsal striatal DA level.

	n	Mean (pg/µl)	SEM		*n*	Mean (pg/µl)	SEM
Baseline				Peak			
Dorsal Striatum				Dorsal Striatum			
E2	9	0.462	0.101	E2	9	2.138	0.304
CYC	8	0.853	0.095	CYC	8	3.248	0.253
medial Prefrontal Cortex				medial Prefrontal Cortex			
E2	7	0.337	0.064	E2	7	2.769	0.588
CYC	7	0.360	0.083	CYC	7	3.036	0.556
Substantia Nigra				Substantia Nigra			
E2	8	0.866	0.180	E2	8	6.214	1.208
CYC	9	0.778	0.108	CYC	9	4.668	0.585

Baseline, absolute mean baseline dorsal striatal DA levels prior to any drug administration; Peak, absolute peak dorsal striatal DA levels post AMPH injection and E2/CYC infusion. E2 = 17β-estradiol; CYC = cyclodextrin; SEM = standard error of the mean. No significant differences were observed between groups.

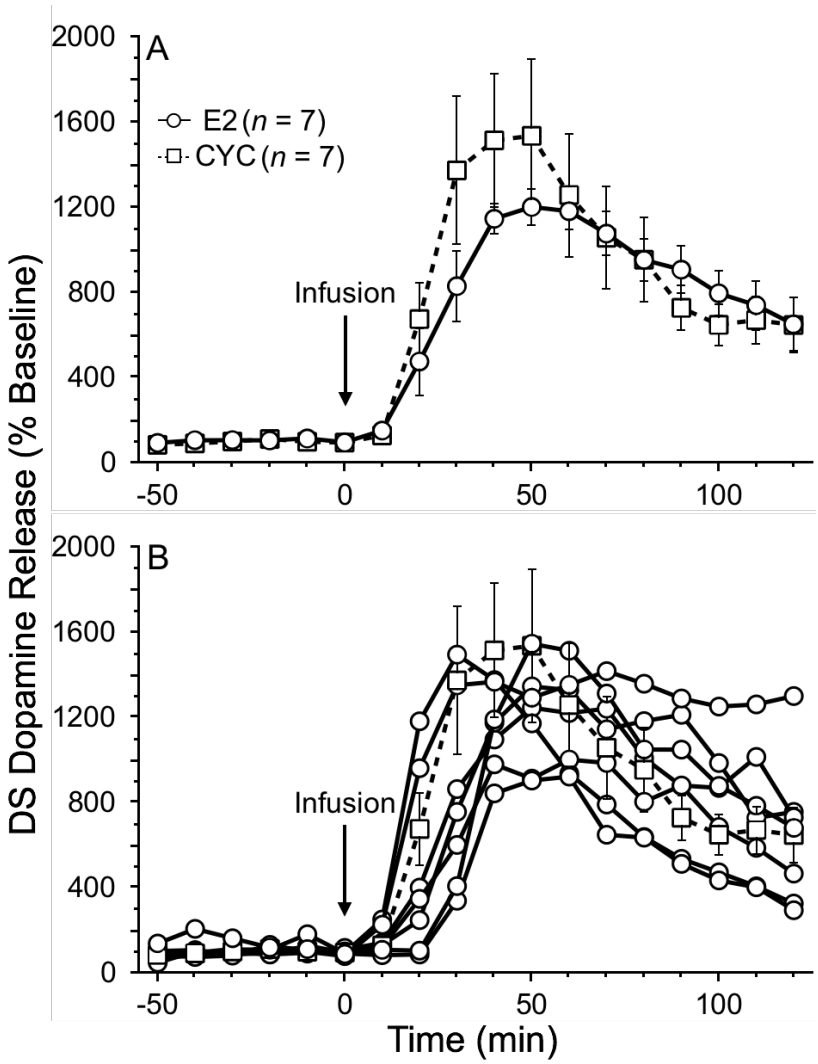

Figure 4. DA in dialysate from DS after E2 or CYC infusion into the mPFC expressed as percentage baseline. Arrow indicates time at which AMPH was administered IP. **A**) Group data; **B**) each individual rat in the E2 group. Error bars represent standard error of the mean. DS = dorsal striatum; E2 = 17β-estradiol; CYC = cyclodextrin; AMPH = amphetamine.

between E2 and CYC (Figure 6C and 6F). DA release was increased in response to AMPH, as reflected by a significant main effect of time ($F_{(11,165)} = 23.898$, $p < 0.001$; $\eta_p^2 = .614$). AMPH-induced DA release did not differ between two the groups ($F_{(1,15)} = 1.215$, $p = 0.288$; $\eta_p^2 = .075$; *see* Figure 5A), E2 and CYC respectively. The findings of this study suggest that infusion of E2 into the SN, the site of DA neuron cell bodies, does not alter AMPH-induced DA release in the DS. However, within the experimental group, when each rat was examined independently, it was noticed that they fell into one of two groups; either high responders, or low responders (*see* Figure 5B). These data suggest that some rats have an increased AMPH-induced DA release in the DS, while others have a decreased AMPH-induced DA release in response to E2 infusion into the SN, in comparison to the CYC group.

No statistically significant differences in DA metabolites, DOPAC and HVA, were observed (Figure 6).

4. Discussion

In Experiment 1, AMPH-induced DA release was shown to be significantly higher in the E2 group when E2 or CYC was directly infused into the DS (*see* Figure 5A). This finding is the first to indicate that direct infusion of E2 into the DS rapidly increases AMPH-induced DA release *in vivo*, similar to Becker's finding *in vitro* (1990a, 1990b). These findings support our hypothesis that E2 acts directly within the DS to modulate DA transmission and previous work showing that E2 modulates DA in the DS following systemic injections (Di Paolo et al., 1985; Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999). It is still not yet known how E2 may be having such effects; they may be mediated by ERs in the DS. We have shown via electron microscopy that ERα, ERβ, and GPER-1 are localized at the membrane of cells within the DS (Almey et al., 2012). Moreover, these receptors are rarely observed in the nucleus. Specifically, ERs were most frequently observed in glia and in the axons and terminals of cholinergic (Almey et al., 2012) and GABAergic (Almey et al., 2016) neurons. Notably, ERs were not observed on DA terminals. Schultz and colleagues (2009) showed *in vitro* that the potentiated DA response to E2 involves GABA and Mermelstein et al (1996) showed that E2 acts directly on the membrane of GABA medium spiny neurons. It has also been shown that estrogens rapidly affect D2R binding (Bazzett and Becker, 1994). Thus, the rapid effects of E2 on DA release in the DS is

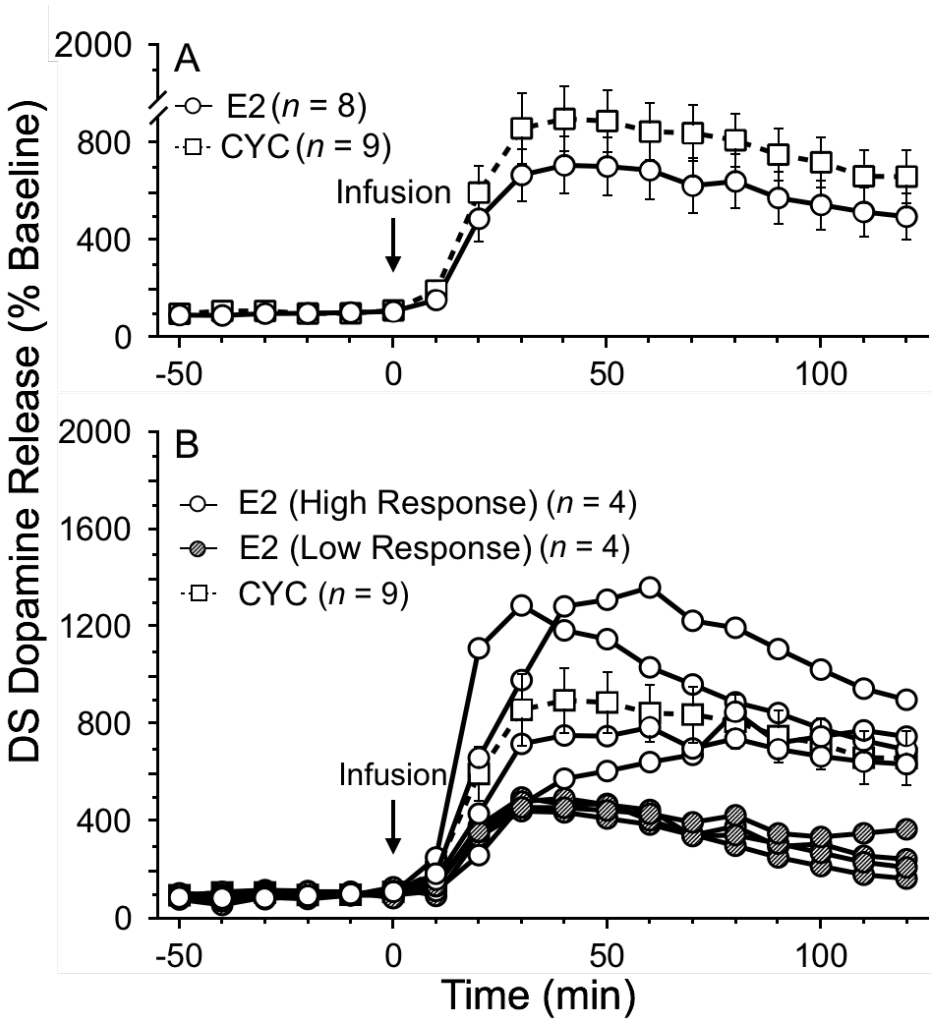

Figure 5. DA in dialysate from DS after E2 or CYC infusion into the SN expressed as percentage baseline. Arrow indicates time at which AMPH was administered IP. **A**) Group data; **B**) each individual rat in the E2 group. Error bars represent standard error of the mean. DS = dorsal striatum; E2 = 17β-estradiol; CYC = cyclodextrin; AMPH = amphetamine.

likely a result of it acting on membrane-associated receptors on either GABA or acetylcholine neuron terminals or both.

D2Rs in the DS can exist in either a high or low affinity state for DA and D2R agonists (Sibley et al., 1982). Di Paolo and colleagues (1988) found that fluctuations of hormones across the estrous cycle affect the affinity state of D2Rs such that during periods of high E2, there is a decrease in D2Rs in the high affinity state. Similarly, there is a rapid conversion from high to low striatal D2R affinity state following an acute dose (100ng) of E2 in OVX rats (Levesque and Di Paolo, 1988). Collectively, these results indicate that E2 is rapidly affecting the functionality of D2Rs suggesting that E2 rapidly affects both DA release and D2R function in the striatum.

While there was no significant effect when the means of the two conditions were compared in either the mPFC and the SN groups, an interesting pattern emerged in the latter group when the rats were examined individually. In Experiment 3, half the rats (n = 4) responded to the E2 infusion into the SN by exhibiting a higher DA response, while the other half (n = 4) showed a lower response, when compared to the CYC group (*see* Figure 5B). It is possible that our current findings are due to activation of two subpopulations of substantia nigral DAergic neurons. Chiodo and Caggiula (1983) found that an acute systemic administration of E2 to OVX rats rapidly increased spontaneous activity in "Type A" nigral neurons, but decreased spontaneous activity in "Type B" nigral neurons. They suggested that the effects of E2 on nigral DAergic neurons are not simply an either/or excitatory or inhibitory influence, but rather a more complex bidirectional modulatory influence (Chiodo et al., 1981). Similar to Chiodo and colleagues, this finding also indicates that there are two types of nigral DAergic neurons with opposite effects in response to E2. Based on these findings, it is possible that the effects of E2 on AMPH-induced dorsal striatal DA release measured in the current study was a response of Type A nigral DAergic neurons in half of the rats, and response of Type B nigral DAergic neurons in the other half. As previously indicated, Becker and colleagues have shown a potentiated DA profile in response to systemic E2 administration (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999). Thus, it seems that E2 is acting on several brain regions in concert when injected systemically, and some of these neurons or areas may in fact be inhibitory.

One caveat to be noted is that here DA was measured in anesthetized rats and the results could be influenced by Isoflurane. Future studies should investigate the subpopulation of

DAergic neurons in the SN and the mechanism by which E2 affects each of the two types of neurons separately. Another caveat with regard to these findings is that there are different levels of ERs in each of the brain regions examined here. Thus, the lack of statistically significant effect in the mPFC or the SN could be attributed to the dose of E2 used in this study. Future studies should examine a dose response of E2 within specific brain regions.

In conclusion, the present study demonstrated that local infusions of E2 into the DS result in an AMPH-induced dorsal striatal DA release *in vivo*. This effect was not seen when E2 was infused into the mPFC or the SN. When examined individually, the rats in Experiment 3 revealed two types of responders, those that show a potentiated DA response to SN E2 infusions, and those that show a reduced DA response. These data further elucidate the complex functions of E2 in the brain on dorsal striatal DA transmission. They provide evidence that E2 may be acting directly in the DS to affect multiple memory system bias in females. Understanding such effects is also important to help resolve the role of ovarian hormones in disorders such as schizophrenia and Parkinson's disease.

CHAPTER 3:
17β-ESTRADIOL LOCALLY INCREASES PHASIC DOPAMINE RELEASE IN THE DORSAL STRIATUM.

Waqqas M. Shams, Marie-Pierre Cossette, Peter Shizgal, and Wayne G. Brake

Citation: Neuroscience Letters (2017); *in press.*

Preface:

In Chapter 2 we found that E2 can increase tonic striatal DA release in anesthetized female rats. However, it is not known if E2 can also increase phasic striatal DA release. Therefore, here, we measure the effects of E2 on phasic dorsal striatal DA release in anesthetized female rats. The methodology in this study was adjusted to effectively detect phasic DA release. To measure DA release, the SN was electrically stimulated and fast-scan cyclic voltammetry was used to detect phasic DA transmission.

Abstract:

Studies using *in vivo* microdialysis have shown that E2 increases DA transmission in the DS. Both systemic administration of E2 and local infusion into the DS rapidly enhance AMPH-induced DA release. However, it is not known to what degree these effects reflect tonic and/or phasic DA release. It was hypothesized that E2 acts directly within the DS to rapidly increase phasic DA transmission. In urethane-anaesthetized (1.5mL/kg) female rats, we used fast-scan cyclic voltammetry to study the effects of E2 on phasic, electrically-evoked release of DA in the DS. Rats were OVX and implanted with a silastic tube containing 5% E2 in cholesterol, previously shown to mimic low physiological serum concentrations of ~ 20-25 pg/ml. DA release was evoked every 1 min by delivering biphasic electrical stimulation in the SN. Local infusions of E2 (244.8 pg/µl) into the DS increased the amplitude of the electrically evoked DA transients. Behaviorally significant stimuli and events trigger phasic release of DA. The present findings predict that E2 would boost such signaling in behaving subjects.

1. Introduction

Striatal DA transmission is important for many functions including cognition, motor control, movement, learning, motivation, and reward (Bannon et al., 2012). Such functions are also influenced by E2. It is well established that estrogens modulate DA signaling in the DS (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999). E2 is the most highly concentrated circulating estrogen during reproductive years in females (Gillies and McArthur, 2010), and replacement of this hormone in OVX rats rapidly (within 30 min) restores and enhances AMPH-induced striatal DA release (Becker and Beer, 1986; Becker, 1990a, 1990b; Castner et al., 1993; Becker and Rudick, 1999). Initially, such a rapid increase in striatal DA was shown in *in-situ* slice preparations or with systemic injection of E2 (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999). Recently, we have shown via *in-vivo* microdialysis that local infusions of E2 into the DS also result in a greater AMPH-induced release of DA in the DS (Shams et al., 2016).

Release of DA varies along a continuum of timescales (Schultz, 2000) ranging from sustained "tonic" to episodic "phasic" release (Grace, 1991). Tonic DA release maintains concentrations of extracellular DA that depend, among other influences, on baseline firing. Phasic DA release, on the other hand, is driven by burst firing and occurs in response to behaviorally salient stimuli (Grace, 1991). The temporal resolution of the *in-vivo* microdialysis method is low. Thus, to determine at what points along the tonic-phasic continuum E2 modulates DAergic neurotransmission in the DS, it is necessary to complement the existing microdialysis data with observations obtained by means of a method with higher temporal resolution.

Although the local effect of estrogens on phasic DA release in the DS has not been reported previously, the effects of estrogens on DA release in the NA (Thompson and Moss, 1994) and the central nucleus of the amygdala (Liu and Xie, 2004) have been examined using *in vivo* voltammetry. Direct infusion of E2 into the NA results in a rapid increase in phasic K^+-stimulated DA release (Thompson and Moss, 1994), whereas subcutaneous estradiol benzoate (another estrogen) increases electrically-evoked phasic DA release in the central nucleus of the amygdala (Liu and Xie, 2004) in OVX rats. In the current study, the SN was electrically stimulated and striatal phasic DA release was measured in response to a local infusion of E2 or

its vehicle (cyclodextrin) in the DS. Phasic DA release was measured using fast-scan cyclic voltammetry (FSCV) in anesthetized OVX female rats. It was hypothesized that E2 acts directly within the DS to rapidly increase phasic DA transmission.

2. Methods

2.1 Animals

Female Long-Evans rats (Concordia University in-house breeding colony) weighing 220-260 g and 60-65 days old, were single housed at 21°C in polypropylene shoebox cages with beta-chip bedding. A 12:12 h reverse light/dark cycle was used with lights off at 9:00 AM. Rats had access to *ad libitum* Purina rat chow and water. All handling, testing and surgical procedures were performed during the dark phase of the diurnal cycle. All protocols were previously approved by Concordia University's animal research ethics committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Surgery

In preparation for the ovariectomy surgery, twelve rats were anesthetized using Isoflurane (4% for induction; 2% for maintenance: Inhalation Anesthetic, Richmond Hill, ON, Canada) and bilaterally OVX through a dorsal incision (1 cm). Post-ovariectomy, all rats were implanted with a silastic tube (1 cm long: 1.96 mm OD, 1.47 mm ID) containing 5% E2 (Sigma Chemical Co., St. Louis, MO, USA) in cholesterol (Sigma) in the nape of the neck. The silastic tube containing E2 was previously shown to mimic low physiological serum concentrations of ~ 20 – 25 pg/mL consistent with estrus (Almey et al., 2013). The purpose of such a low chronic E2 administration was to keep the E2 receptors upregulated so as to maintain their sensitivity to later microinfusions of E2. These capsules have been shown to produce consistent E2 serum levels up to 4 weeks following implantation (Almey et al., 2013). For post-surgical care, all rats were administered the anti-inflammatory drug, Anafen (0.1 mL/rat, SC; MERIAL Canada Inc., Morgan Baie d'Urfe, QC, Canada) and the antibiotic Penicillin G (0.2 mL/rat, intramuscular; CDMV, St. Hyacinthe, QC, Canada); antibiotic ointment (By Pharmaceuticals Inc., Brampton, ON, Canada) was applied to the incision. Following surgery, all rats were allowed at least one week to recover in their home cages. The rats were then pseudorandomly assigned to one of the two groups, E2 (n = 6) or CYC (n = 6) and were tested between days 8 and 21 post ovariectomy.

2.3 FSCV recordings

Each rat was anesthetized using urethane (1.5 mL/kg, IP), given an injection of atropine sulphate (0.05 mg/kg SC; Sandoz Canada Inc., QC, Canada) to reduce bronchial secretions during surgery, and given an injection of saline (1.0 mL SC; Baxter, Baxter Corporation Mississauga, ON) every 2 hours to prevent dehydration. Prior to placing the rat in the stereotaxic apparatus (David Kopf Instruments; Tujunga, CA, USA), tear gel (Alcon Canada Inc., Mississauga, ON, Canada) was applied to the surface of the eyes, to avoid dryness, and the topical anesthetic, xylocaine (AstraZeneca Canada Inc., Canada), was applied to the external auditory meatus of the rat to reduce discomfort from the ear bars. The two stimulation electrodes were stereotaxically implanted, secured with dental acrylic, and anchored with jeweler's screws.

The stimulating electrode, consisting of a 0.25 mm stainless steel insect pin was insulated with Formvar enamel to within 0.5 mm of the tip. It was aimed at the following coordinates in the right SN (referenced to bregma); AP: -4.8 mm; ML: 2.4 mm; DV: -7.9 mm (Paxinos and Watson, 2007). A second electrode was made in the same manner as the first, but 3 mm of insulation was stripped at the tip. This electrode was aimed at the lateral orbital cortex (AP: 4.3 mm; ML: 2.4 mm; DV: -5.5 mm) to serve as the anode of the stimulation circuit. The coordinates for the anode were chosen in a similar manner to previously reported methods (Cossette et al., 2016). Briefly, the placement of the anode was chosen to place the recording site mid-way between the cathode (stimulating electrode) and the anode of the stimulation circuit. The reference electrode was then placed on or near the zero potential surface of the electrical field around the stimulation current to reduce electrical artifacts in the voltammetric recordings.

2.4 Hormone administration

During the experiment, all rats received a local (1.0 µl) intracerebral microinfusion, at a rate of 0.2 µl/min, of either the E2-CYC complex (244.8 pg/µl; Sigma), or the vehicle, CYC (5.196 ng/µl; Sigma), mixed in sterile saline (Hospira, IL, USA). E2 was encapsulated in CYC to make it water-soluble. We have shown this dose of E2 to be effective when infused into the DS to increase AMPH-induced striatal DA release (Shams et al., 2016) and to alter memory system bias when infused into the mPFC (Almey et al., 2014).

2.5 Voltammetry

The carbon fiber (Thorne, Amoco Corporation, Greenville, SC, USA) was glass-encased, and a seal was produced by heating the glass capillary with a pipette puller (PUL-1, WPI, Sarasota, FL, USA). A wire covered with silver paint (GC Electronics, Rockford, IL, USA) was

inserted in the capillary to make contact with the carbon fiber and secured with shrink tubing coated with epoxy (150-200 μm exposed tip length, 7 μm diameter). A 21 Ga stainless steel tube (30 mm in length) was secured with shrink tubing to the carbon fiber electrode and spaced 1 mm laterally and 1 mm above the exposed carbon fiber. This tube served as an injector needle holder. The carbon fiber electrode (working electrode) was aimed at the DS (AP: -0.3 mm, ML: 3.5 mm, DV: -4.0 mm – -5.6 mm). FSCV was computer-controlled as described previously (Heien et al., 2003). Briefly, background-subtracted cyclic voltammograms were generated at 10 Hz by applying an 8.5 ms triangular waveform that ramped from -4.0 V to +1.3 V and back to -0.4 V at a scan rate of 400 V/s. The potential was held at -0.4 V between scans to promote cation absorption at the surface of the FSCV electrode. All potentials were measured with respect to the Ag/AgCl reference electrode. The waveform was generated using LabVIEW (National Instruments, Austin, TX) and a multifunction data acquisition board (PCI-6052E, National Instruments, Austin, TX).

A PCI-6711E board (National Instruments, Austin, TX) was used to perform waveform acquisition, and data collection. A synchronization signal from the PCI-6711E board was sent to the external input of a multi-channel pulse generator (Master-8, A.M.P.I. Jerusalem) and used to trigger the electrical stimulation 5 s after the start of each recording. The stimulation was patterned to prevent overlap with the voltammetric scans. This was accomplished by confining pulse generation to the 91.5 ms intervals separating the triangle waves. Voltages generated by the Master-8 pulse generator were converted to constant currents via a stimulus isolation unit (AM-2200, AM-Systems, Carlsborg, WA).

Once the carbon fiber electrode was in place, six baseline voltammograms were collected. DA release was evoked every 1 min by delivering electrical stimulation in the SN (500 ms trains duration, 2 ms pulse duration, 200 – 400 μA, 60Hz). Following collection of the baseline voltammograms, an intracerebral microinfusion of either E2 or CYC was administered at a rate of 0.2 μl/min to yield a total volume of 1.0 μl. The injector remained in place for the duration of the collection period to prevent any additional disruption close to the recording site. Immediately at the end of the infusion, four blocks of six voltammograms were collected, with a 5 min inter-block interval. During the inter-block intervals, the carbon fiber was cycled at 60 Hz to maintain sensitivity to changes in DA concentration.

2.6 Statistical Analyses

The electrically evoked DA transients obtained during baseline testing were compared to electrically evoked DA transients recorded following vehicle or E2 infusion. To render these responses comparable, the peak of each DA transient was normalized to its respective averaged baseline. This was done by dividing each peak by the average of the six baseline peaks. Matlab (Natick, MA, USA) was used to perform resampling with replacement (bootstrapping with 1000 iterations) so as to assess the statistical reliability of the effects (Efron and Tibshirani, 1993). The criterion for a statistically reliable effect in the resampling analysis was an absence of overlap between the 95% confidence intervals around the results from the E2 and vehicle conditions.

2.7 Histology

After the completion of the experiment, a lethal injection of sodium pentobarbital (120 mg/rat, IP) was administered. The location of the stimulating and recording electrodes was marked by passing 1 mA of anodal current for 20 s. The animals were then perfused intracardially with 0.9% sodium chloride, followed by a formalin-Prussian Blue solution (10% formalin, 3% potassium ferricyanide, 3% potassium ferrocyanide, and 0.5% trichloroacetic acid) that forms a blue reaction product from the iron deposited at the tip of the stimulating electrode. Next, the brains were removed and fixed with 10% formalin solution. 40 μm thick, coronal sections were cut with a cryostat (Thermo Electron Corporation) and examined to confirm placements of the FSCV electrode and injector in the DS and the stimulating electrode in the SN (*see* Figure 1).

3. Results

DA transients were time-locked to the electrical stimulation, and all voltammograms had a signature characteristic of DA, with an oxidation peak at roughly 0.65 V and a reduction peak at roughly -0.2 V (*see* Figure 2 for a representation of DA transients prior to and following E2 or CYC administration).

The peaks of the DA responses following E2 infusion were systematically higher than the peaks in the corresponding baseline voltammograms (*see* Figure 3A) and were maximal by the end of the 5-min infusion. There is no overlap between the 95% confidence intervals surrounding the DA responses observed during the baseline, thus meeting our criterion for a statistically reliable effect. In contrast, no differences were found between the peaks of the DA responses following CYC infusion and the corresponding baseline voltammograms (*see* Figure 3B).

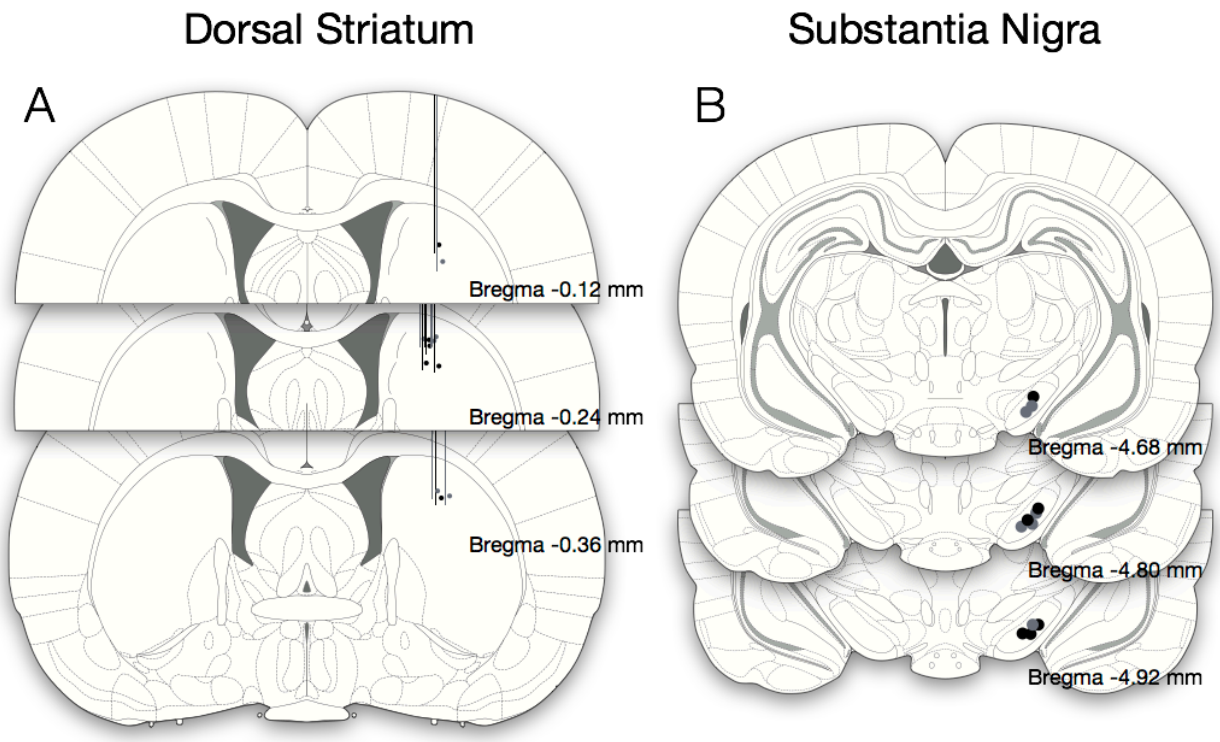

Figure 1. Schematic representation of (**A**) carbon fiber and injector placements in the DS and (**B**) electrode placements in the SN. Black lines represent all E2 group carbon fiber placements, while grey lines represent all CYC group carbon fiber placements. Black circles represent all E2 group injector or electrode placements, while grey circles represent all CYC group injector or electrode placements. E2 = 17β-estradiol; CYC = cyclodextrin.

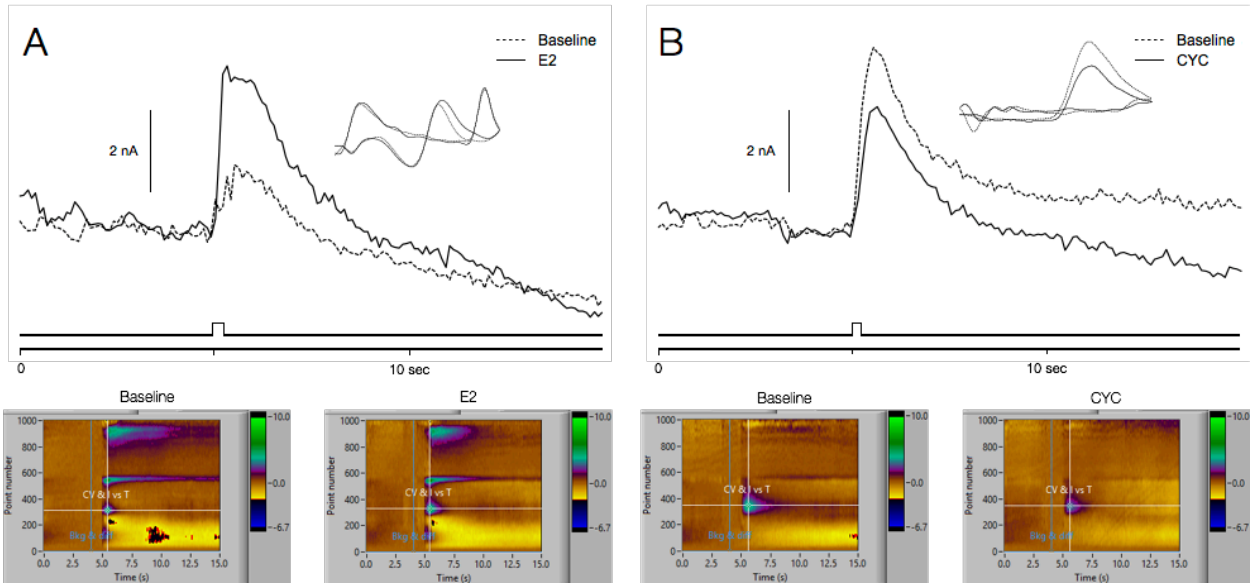

Figure 2. Dorsal striatal DA transients prior to and following drug administration. **A**) DA traces prior to and following E2 administration. **B**) DA traces prior to and following administration of the vehicle, CYC. E2 = 17β-estradiol; CYC = cyclodextrin.

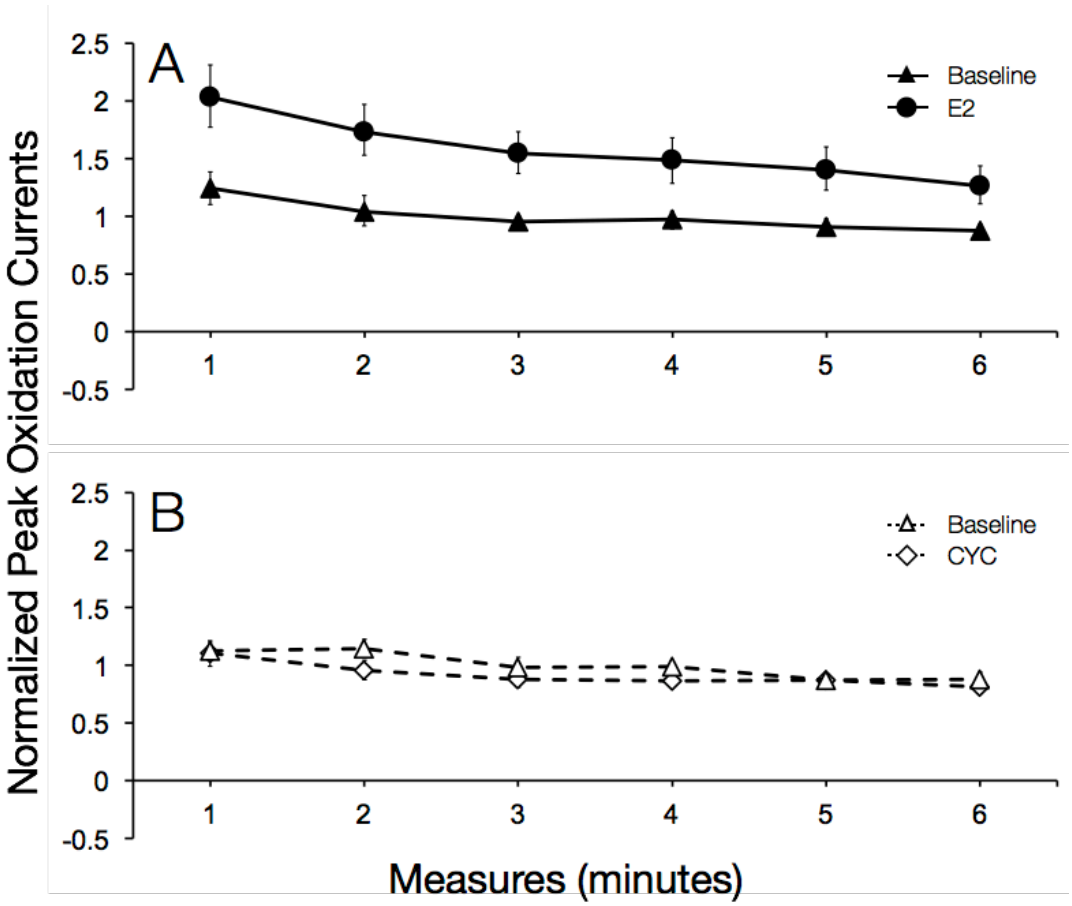

Figure 3. Dorsal striatal DA concentrations in response to E2 or the vehicle, CYC, administration. **A**) Response to E2, and **B**) CYC administration in comparison to baseline, normalized using all baseline measures. It should be noted that error bars in panel B are smaller than the symbols. Error bars represent 95% confidence intervals around the mean. E2 = 17β-estradiol; CYC = cyclodextrin.

4. Discussion

Infusions of E2 increased the amplitude of electrically-evoked DA transients in the DS, as measured by FSCV, whereas the CYC vehicle was without effect. These findings support our hypothesis that E2 acts directly within the DS to rapidly increase phasic DA transmission. In previous studies employing microdialysis (Becker and Cha, 1989; Becker, 1990b; Castner et al., 1993; Xiao and Becker, 1994; Becker and Rudick, 1999; Shams et al., 2016), increases in the extracellular concentration of DA in the DS were observed, but due to the low temporal resolution of the measurement method, it was not possible to determine the degree to which changes in tonic or phasic release were responsible. The present results suggest that potentiation of phasic DA release by E2 could have contributed to the changes in DA concentration observed previously in microdialysis samples following administration of E2 to the DS. To our knowledge, this study is the first to show that direct infusion of E2 into the DS increases phasic DA release *in vivo*.

The effect of E2 on phasic DA release was maximal by the end of the 5-min infusion. This short latency suggests that E2 must have acted non-genomically in the DS. Electron microscopy has shown that ERα, ERβ, and GPER-1 are localized at the membrane of neurons within the DS (Almey et al., 2012). These receptors are rarely found in the nuclei of neurons within the DS. In fact, striatal ERs are most frequently observed in the axons and terminals of GABAergic (Almey et al., 2016) and cholinergic neurons, as well as in glia (Almey et al., 2012). Interestingly, ERs are not seen on DA terminals in the DS. However, $GABA_B$ receptors are located on presynaptic DA terminals, and play a critical role in the inhibition of striatal DA release (*for review, see* Schultz et al., 2009). Given that systemic injections of E2 rapidly reduce striatal GABA concentrations (Hu et al., 2006), the presence of E2 in the DS could indirectly increase striatal DA release through attenuation of GABA release. Such effects could be mediated by membrane-bound ERα on medium-spiny GABA neurons (Schultz et al., 2009). Supporting evidence is provided by recent findings by our laboratory showing that ERs are present on the membrane of GABAergic neurons (Almey et al., 2012, 2016). Thus, the rapid effects of E2 on phasic DA release in the DS reported here could be due to E2 action at membrane-associated ERs on the terminals of GABA neurons. Membrane-associated ERs are also found on cholinergic neurons in the DS which could also have contributed.

The findings of the current study show that in addition to any effects of E2 on tonic DA signaling in the DS, E2 boosts phasic DA signaling. These results have implications for the influence of estrogens on processes dependent on phasic DA signaling, such as learning, movement, and motivation.

CHAPTER 4:
PHARMACODYNAMIC EVIDENCE FOR SEX DIFFERENCES IN RESPONSE TO ANTIPSYCHOTICS

Waqqas M. Shams, Marc Morissette, Anne Almey, Alison S. Fleming, Thérèse Di Paolo, and Wayne G. Brake

Submitted.

Preface:

Chapters 2 and 3 investigated the effects of E2 on tonic and phasic dorsal striatal DA release. However, the mechanism through which these effects occur remains unknown. Therefore, we further examined the effects of E2 on DA transmission in conjunction with HAL. Specifically, in Chapter 4, we investigated the effects of E2 and HAL on DA transmission through their effects on the density and affinity state of D2Rs in AMPH-sensitized female and male rats via receptor binding.

Abstract:

Administrating estrogens with antipsychotics is a beneficial adjunctive therapy for women with schizophrenia, but the reasons why are unknown. Antipsychotics primarily target D2Rs. D2Rs have two affinity states, i.e. $D2^{High}$ and $D2^{Low}$, and these affinity states are affected independently by both HAL and estrogens. The combined effects of the antipsychotic, HAL, and E2, on brain pharmacodynamics in females is not known. Little is known about the pharmacodynamics of HAL in males as well. Here, thirty-six female rats were OVX and received either no, low, or high E2 replacement, while eighteen male rats received a sham surgery. All rats were AMPH-sensitized and were administered either chronic HAL or saline. D2R binding affinities were then examined in the DS. HAL increased total D2R binding in no E2 and low E2, but not high E2, replacement rats, while both HAL and E2 increased the affinity of $D2^{High}$ and $D2^{Low}$ receptors. Additionally, HAL and E2 administered together decreased the proportion of D2Rs in the high affinity state. Chronic HAL did not have an effect on total binding, affinity, or proportion of D2Rs in the high affinity state in AMPH-sensitized male rats. These findings demonstrate that sex differences in response to HAL treatment are, in part, due to receptor function, specifically the affinity of the D2R, and are also dependent on circulating levels of estrogens.

1. Introduction

Recent evidence suggests that estrogens administered in conjunction with antipsychotics are a better form of therapy for women suffering from schizophrenia than antipsychotics alone. Several studies found that estrogen levels have a direct inverse correlation with the dose of antipsychotics, such that as estrogen levels increase, women with schizophrenia require lower antipsychotic dose, and vice versa (Hallonquist et al., 1993; Gattaz et al., 1994; Riecher-Rössler et al., 1994). Women receiving estrogen therapy in conjunction with antipsychotics have a better response to treatment than those treated with antipsychotics alone (Kulkarni et al., 1996, 2001; Akhondzadeh et al., 2003; *for review, see* Kulkarni et al., 2012). These findings provide some evidence that gonadal hormones, like estrogen, modulate the severity of schizophrenia and the effects of antipsychotics. One obvious reason for such sex differences in response to antipsychotics is pharmacokinetics (Seeman, 2004). It is proposed here that the influence of estrogens on responsivity to antipsychotics also may be through their effects on pharmacodynamics in the brain.

D2Rs have two affinity states, $D2^{High}$ and $D2^{Low}$ (Wreggett and Seeman, 1984). D2Rs are functional when they are in the high affinity state ($D2^{High}$; George et al., 1985) and are functionally inert when they are in the low affinity state ($D2^{Low}$; Seeman, 1982). There is a greater density of $D2^{High}$ present in the DS and NA of unmedicated individuals with schizophrenia than there are in individuals without schizophrenia (Seeman 1987). Although, in the striatum, 15 – 20% of D2Rs are $D2^{High}$, in other areas, about 90% are $D2^{High}$ (*for review, see* Seeman, 2006). Seeman et al. (2005) showed that repeated administration of AMPH leads to DA supersensitivity. In such cases, the density of D2Rs does not change, however, the proportion of $D2^{High}$ increases significantly (Seeman et al., 2005). Importantly, Seeman (2009) found that HAL treatment changed 60% of $D2^{High}$ into $D2^{Low}$ in the striatum of AMPH-sensitized rats. It is postulated that this change in the affinity state of the D2Rs is partially responsible for the clinical effects of this drug.

The affinity state of D2Rs can be altered by estrogens. For example, the affinity state of D2R fluctuates across the estrous cycle of a rat with the most $D2^{High}$ during diestrus 2 and the most $D2^{Low}$ during the estrus and proestrus (Di Paolo et al., 1988). In addition, chronic estradiol replacement in OVX rats results in an increase in striatal D1R binding suggesting that estradiol affects both the affinity state of D2Rs and the binding density of D1Rs (Levesque and Di Paolo,

1989). Similarly, an acute dose (100 ng) of E2 rapidly converts striatal $D2^{High}$ into $D2^{Low}$ in OVX rats (Levesque and Di Paolo, 1988).

AMPH-sensitization has been used as a rat model of some of neurochemical and behavioral changes seen in schizophrenia (Magos, 1969; Echols, 1977; Mamelak et al., 1985; Robinson et al., 1985; Cador et al., 1995; Vezina, 1996). Recently, we looked at the effects of E2 and whether it can help facilitate the effects of antipsychotics (HAL) on cognitive deficits in AMPH-sensitized rats. A 0.25 mg/kg/day dose of HAL improves perseveration and reversal learning when AMPH-sensitized OVX rats were given low E2 replacement, while 0.13mg/kg/day dose of HAL improves reversal learning when AMPH-sensitized OVX rats were treated with high E2 (Almey et al., 2017). Also, high E2 replacement helps facilitate HAL (0.05 mg/kg, IP) in restoring latent inhibition in OVX rats (Almey et al., 2013). Lastly, AMPH-sensitized OVX rats with high E2 replacement treated with chronic HAL display reduced AMPH-induced accumbal DA release and locomotor activity (Madularu et al., 2014).

Thus, it was hypothesized that HAL and E2 treatment may affect D2R affinity state in an interactive manner. AMPH-sensitized OVX female rats underwent HAL or saline treatment, receiving no, low, or high E2 replacement. In addition, the D2R affinity state in AMPH-sensitized male rats was also measured to see if HAL treatment may be associated with any sex differences on D2R affinity state. It was thought that HAL treatment and E2 replacement together may have combined effects in changing D2R affinity state. It was hypothesized that one mechanism whereby E2 may enhance the effects of HAL on behavior and DA release, as seen in previous studies in our laboratory, is by changing the affinity state of D2Rs in the striatum.

2. Methods

2.1 Animals

Male and female Long-Evans rats (Charles River Laboratories, St. Constant, QC, Canada) weighing 250-350 g were single housed at 21°C in polypropylene shoebox cages with beta-chip bedding. A 12:12 h reverse light/dark cycle was used with lights off at 9:00 AM. Rats had access to *ad libitum* Purina rat chow and water. All handling, testing and surgical procedures were performed during the dark phase of the diurnal cycle. All protocols were previously approved by Concordia University's animal research ethics committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Experiment 1

Seventy-eight female rats and twenty-four male rats were repeatedly administered intraperitoneal injections to induce locomotor sensitization. Once AMPH-sensitized, a subset of the female and male rats were then used in Experiment 2 to examine D2R binding affinities in the DS.

2.3 Experiment 2

2.3.1 Surgery

Thirty-six female rats were anesthetized using Isoflurane (4% for induction; 2% for maintenance; Inhalation Anaesthetic, Richmond Hill, ON, Canada) and bilaterally OVX through a dorsal incision (1 cm). Eighteen male rats received a sham ovariectomy surgery in a similar manner. Following ovariectomy, 24 female rats were implanted with an E2 silastic tube (1 cm long; 1.96 mm OD, 1.47 mm ID) in the nape of the neck, while 12 female and 18 male rats received a sham capsule implantation surgery, but no actual capsule was implanted. For post-surgical care, all rats were administered the anti-inflammatory drug, Anafen (0.1 mL/rat, SC; MERIAL Canada Inc., Morgan Baie d'Urfe, QC, Canada), the antibiotic Penicillin G (0.2 mL/rat, intramuscular; CDMV, St. Hyacinthe, QC, Canada) and antibiotic ointment (By Pharmaceuticals Inc., Brampton, ON, Canada) was applied to the incision. Following ovariectomy, all rats were given one week to recover in their home cages and the female rats were divided into 3 hormone condition groups; no E2 (n = 12), low E2 (n = 12), and high E2 (n = 12).

2.3.2 Hormone replacement and HAL treatment

Female rats in each of the three hormone conditions, and the male rats, were randomly divided into two drug treatment groups, HAL or saline. The HAL group (n = 27; 0.25 mg/kg/day; Sandoz Canada Inc., QC, Canada) was implanted with Alzet osmotic minipumps (model: 20002; Durect, Cupertino, CA, USA) that released HAL at a rate of 0.5 μL/h across 14 days, while the saline group (n = 27; 0.9%) were implanted with the same minipumps that released saline in a similar manner. Delivery of 0.25 mg/kg/day of HAL via minipump has been shown to produce a steady state D2 receptor occupancy in rats, closely approximating the pharmacokinetic profile of effective drug doses observed in humans (Samaha et al., 2007, 2008).

Low E2 was administered via silastic capsule containing 5% E2 (Sigma Chemical Co., St. Louis, MO, USA) in cholesterol (Sigma). These capsules containing E2 have previously

shown to mimic low physiological serum concentrations of ~20 – 25 pg/ml consistent with estrus (Almey et al., 2013). In addition to the capsules, rats in the high E2 group received injections of E2 in sesame oil once every four days (10 µg/kg, SC), following the minipump implantation. We have shown that the combination of the E2 capsule and SC E2 injection achieves an average plasma level of ~90 pg/ml across 12 hours (Almey et al., 2013), similar to average plasma levels of E2 consistent with the proestrus phase of the estrous cycle (Butcher et al., 1974). Female rats in the no E2 and low E2 groups and all male rats received sesame oil injections.

2.3.3 AMPH sensitization

Habituation. Rats were habituated to the locomotor activity chambers (42 cm x 25 cm x 17 cm) for 2 hr/day for 2 consecutive days. The behavior was monitored for a period of 120 min by recording interruptions of photobeams of two pairs of photocells at 14 cm increments, located 2.5 cm above the grid floor that were interfaced to a computer running a custom designed software. On the second day of habituation, rats received an IP injection of saline 1 day prior to the induction phase of AMPH-sensitization to familiarize them with the injection process.

Induction. Rats received repeated administration of *D*-amphetamine sulphate (1 mg/kg or 0.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% saline via intraperitoneal injection (IP) to induce locomotor sensitization. Rats were administered a single daily injection of AMPH (1 mg/kg, IP) for four consecutive days while locomotor activity was measured (Robinson and Becker, 1986). The monitoring session was divided into pre-injection (30 min) and post-injection (90 min) components, during which total time spent moving was recorded. All rats were tested throughout the experiment in the same respective activity chamber at the same time of day.

Withdrawal and Expression. After a 1-week AMPH withdrawal period, rats were challenged with half the dose of AMPH (0.5 mg/kg IP) to determine whether they exhibited sensitization to the locomotor activating effects of AMPH. Locomotor AMPH sensitization, and thus neurobiological sensitization, was considered to have developed if rats exhibited comparable or higher levels of locomotor activity in response to the challenge dose of AMPH (0.5 mg/kg) as they did to the initial dose of AMPH (1 mg/kg) in induction (*see* Figure 1). This protocol has previously been shown to induce AMPH sensitization of locomotor activity and accumbal DA release (Madularu et al., 2014).

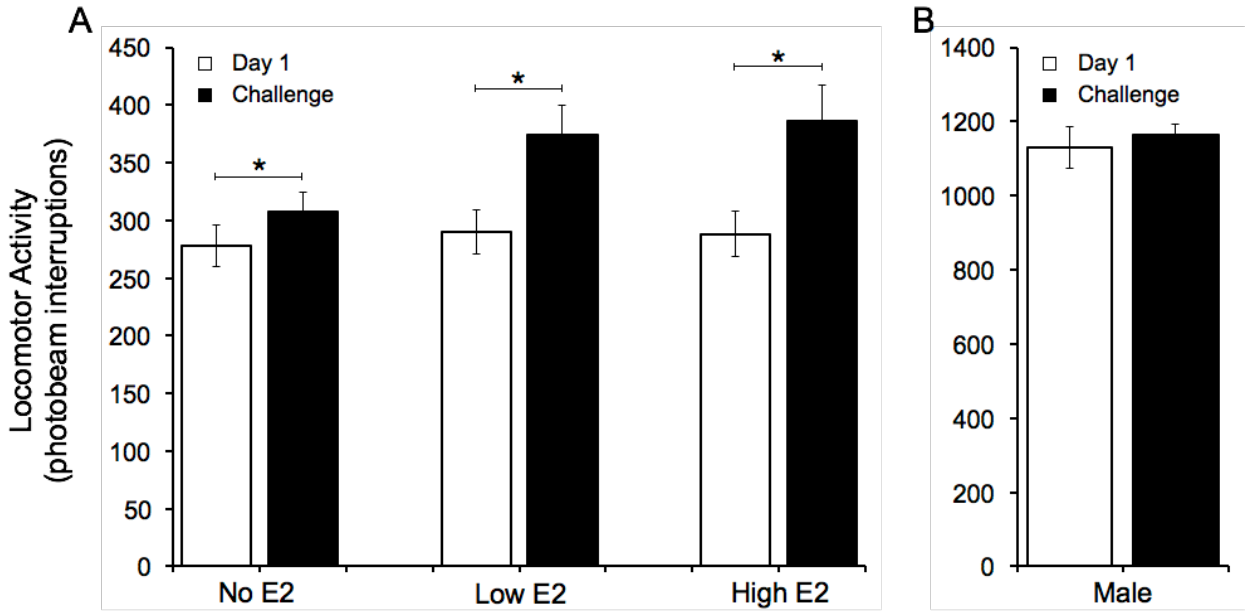

Figure 1. Comparison of mean (± SEM) locomotor activity observed on day 1 of AMPH injections (1.0 mg/kg IP) to the challenge day injection (0.5 mg/kg IP) in A) female and B) male rats following AMPH withdrawal. Equal or greater locomotor activity on challenge day suggests that behavioral sensitization has occurred. E2 = 17β-estradiol. * = $p < 0.05$.

Following AMPH challenge, female rats were further divided into 2 conditions, saline or HAL treated. Similarly, all male rats were also divided into 2 conditions, saline (n = 9) or HAL (n = 9) treated. Rats were anesthetized again and implanted subcutaneously through a dorsal incision, osmotic minipumps containing either HAL or saline. The final group designations were as follows: no E2 saline (NES; n = 6), low E2 saline (LES; n = 6), high E2 saline (HES; n = 6), no E2 HAL (NEH; n = 6), low E2 HAL (LEH; n = 6), high E2 HAL (HEH; n = 6), male saline (MS; n = 9), and male HAL (MH; n = 9; *see* Table 1). Next, the female rats underwent behavioral testing (*for those results, see* Almey et al., 2017).

Upon completion of behavioral testing, on the 12th day after implantation, minipumps were removed and all of the rats underwent a week of HAL or saline withdrawal. Following withdrawal, rats were decapitated and their brains were removed and flash frozen with 2-methylbutane (Isopentane) in dry ice and stored at -80 °C.

2.3.4 D2R binding

The DS from each rat was dissected and homogenized in a motorized teflon-glass homogenizer in 100 volumes (w/v) of phosphate buffer (81 mM Na_2HPO_4, 19 mM KH_2PO_4, 2 mM $MgCl_2$, 0.1% ascorbic acid, pH 7.4, 4 °C) and centrifuged at 40,000 *g* for 15 min. The D2R agonist high- and low affinity states were determined by measuring the competition of [^{3}H]spiperone (0.25 nM; 76.1 Ci/mmol, PerkinElmer, Boston, MA, USA) by DA concentration from 10^{-11} to 10^{-3} M in the presence of 50 nM ketanserin to block serotonin 5-HT_2 receptor binding sites. Each incubation tube was made in (triplicate) to receive in the following order, 300 µl of phosphate buffer, 50 µl of 0.5 µM ketanserin, 50 µl of phosphate buffer containing DA at various concentration or with 50 µl of 10 µM (+)-butaclamol to estimate nonspecific binding, 50 µl of 2.5 nM [^{3}H]spiperone, and finally, 50 µl of tissue homogenate (containing about 15-20 µg of protein). Tubes were incubated for 1 hr at room temperature. The reaction was terminated by rapid vacuum filtration using a 48-well cell harvester (Brandel Gaithersburg, MD, USA) over Whatman GF/C glass fiber filters. This was followed by three rapid rinses (4 ml) with ice-cold phosphate buffer. Bound [^{3}H]spiperone was determined by liquid scintillation spectrometry where the filters were placed in scintillation vials with 7 ml of high-flash liquid scintillation cocktail (Ultima Gold^{TM} MV, Perkin-Elmer, Boston, MA, USA). The vials were incubated overnight and monitored for tritium in a liquid scintillation spectrometer (PerkinElmer, Boston, MA) at 55% efficiency.

Table 1. Schematic representing all of the groups and the treatment they received.

	No E2	**Low E2**	**High E2**	**Male**
Saline	NES (*n* = 6)	LES (*n* = 6)	HES (*n* = 6)	MS (*n* = 9)
Haloperidol	NEH (*n* = 6)	LEH (*n* = 6)	HEH (*n* = 6)	MH (*n* = 9)

All rats were AMPH sensitized and were implanted with either a HAL osmotic minipump (0.25 mg/kg/day) that released HAL at a rate of 0.5 μL/h across 14 days, or a saline osmotic minipump that released saline in a similar manner. E2 = 17β-estradiol; NES = No E2 saline; LES = Low E2 saline; HES = High E2 saline; MS = Male saline; NEH = No E2 HAL; LEH = Low E2 HAL; HEH = High E2 HAL; MH = Male HAL.

Data collected from the liquid scintillation spectrometer was analyzed using the program GraphPad Prism® version 6 (GraphPad Software, Inc., La Jolla, CA, USA). The analysis assumes the binding to be reversible, and that all ligands compete for the same binding sites. The competition data were subjected to a non-linear regression fit using the program GraphPad Prism that provides a statistical approach to determine the best fit between one-site fit and two-site fit models. In this case, Extra sum-of-squares F tests were used to determine the best fit. Significant F tests for all the data identified the two-site model as the best fit, establishing that there were in fact two specific binding sites (i.e., two different affinity states detected from the competition curves). Hence, all of the data presented in the current study were best fitted by a two-site model. Affinity for the two DA sites were 67 nM ($D2^{High}$, 30%), and 14 μM ($D2^{Low}$, 70%) using [^{3}H]spiperone as the radioligand. These two sites were shown to be modulated by E2, guanosine triphosphate (GTP), and sodium (Levesque and Di Paolo, 1988; Di Paolo and Levesque, 1988; Levesque and Di Paolo, 1993). Specifically, Levesque and Di Paolo (1993) have shown that GTP can transform the high affinity site into the low affinity site, thus supporting the functional activity of the sites.

2.4 Statistical analyses

2.4.1 Experiment 1

Dependent samples *t*-tests were conducted to compare day 1 locomotor activity to challenge day locomotor activity to see if rats were AMPH sensitized.

2.4.2 Experiment 2

The effects of E2 administration and HAL treatment were analyzed using a two-way ANOVA with E2 and HAL treatment as a between-factor for each separate measure. When appropriate Tukey's HSD post-hoc analyses were conducted. Independent samples *t*-tests were conducted to measure the differences between HAL and saline treatment in male rats for each separate measure. Planned comparisons were conducted to measure the differences between the control group (NES) and HAL or E2 treatment. Eta squared, partial eta squared, or Cohen's *d* were also calculated to quantify the magnitude of the effect between groups.

3. Results

3.1 Experiment 1

Dependent samples *t*-tests revealed that, in fact, the challenge day locomotor activity was significantly greater than day 1 locomotor activity for no E2 ($t_{(25)} = 1.958$, $p = 0.031$, $d = 0.338$), low E2 ($t_{(25)} = 3.799$, $p < 0.001$, $d = 0.726$), and high E2 ($t_{(25)} = 2.889$, $p = 0.004$, $d = 0.746$) female rats' groups. The dependent samples *t*-test for the male rats' groups was not statistically significant ($t_{(11)} = 0.723$, $p = 0.242$, $d = 0.219$; *see* Figure 1).

3.2 Experiment 2

An example of a competition curve generated from the data shows that approximately 30% of the D2Rs were in the high affinity state, while about 70% of the D2Rs were in the low affinity state (*see* Figure 2).

3.2.1 Total binding (% control) of D2Rs

There was an interaction between drug and hormone treatment ($F_{(2, 30)} = 5.124$, $p = 0.012$, $\eta_p^2 = 0.255$). Tukey's HSD revealed that HAL treatment enhanced total binding of D2R in the NEH group ($p < 0.001$) and the LEH group ($p < 0.001$) in comparison to the non-drug treated controls in AMPH-sensitized female rats. This difference was not observed amongst the high E2 rats. These data show that HAL increased total binding of D2R, but only in the no and low E2 treated groups (*see* Figure 3). There was also a main effect of drug ($F_{(1, 30)} = 88.697$, $p < 0.001$, $\eta_p^2 = 0.747$) and hormone treatment ($F_{(2, 30)} = 4.201$, $p = 0.025$, $\eta_p^2 = 0.219$). HAL had no effect on total binding of D2Rs in AMPH-sensitized male rats ($t_{(15.619)} = 0.003$, $p = 0.998$, $d = -0.001$; *see* Figure 3).

3.2.2 K_i High of D2Rs

There was an interaction between the drug and the hormone treatment ($F_{(2, 30)} = 3.326$, $p = 0.049$, $\eta_p^2 = 0.181$). Tukey's HSD revealed that the control group (NES) had the lowest affinity of $D2^{High}$ in comparison to all other groups (NEH, $p = 0.008$; LES, $p = 0.007$; LEH, $p = 0.001$; HES, $p = 0.027$; HEH, $p = 0.009$) groups (*see* Figure 4). These data show that E2 and HAL treatment increases affinity of $D2^{High}$.

Planned comparisons were conducted for K_i high, K_i low, and proportion of $D2^{High}$. The HAL ($t_{(22)} = 6.443$, $p < 0.001$, $d = 2.605$) and E2 treatment ($t_{(28)} = 4.789$, $p < 0.001$, $d = 2.125$) had significantly higher affinity of $D2^{High}$ in comparison to the control group (*see* Figure 4). These data show that the treatment of HAL and E2, increased affinity of $D2^{High}$ in comparison to

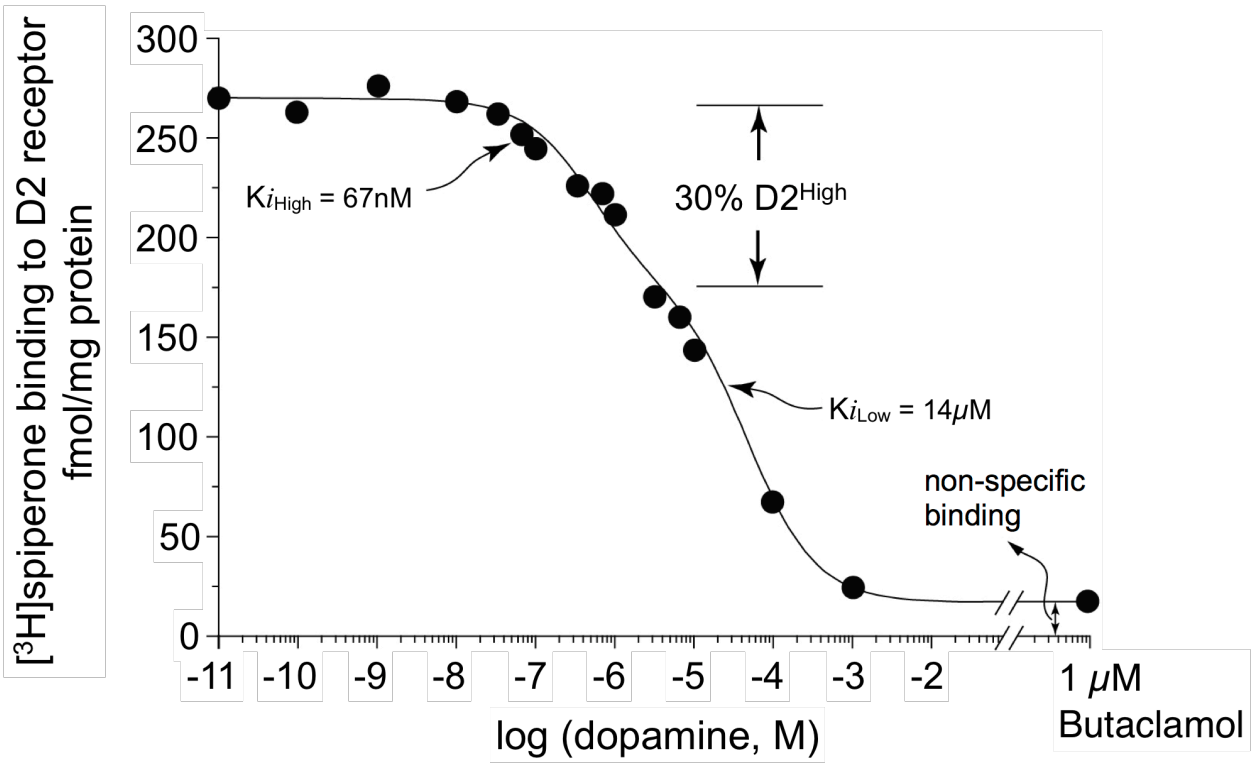

Figure 2. Representative example showing the agonist binding characteristics of D2Rs by determining DA competition for [^{3}H]-spiperone binding in homogenates of the striatum of an intact female rat. (+)butaclamol at 1 μM was used to estimate non-specific binding and represents about 15% of the total binding. Arrows represent high- and low-affinity inhibition constants (Ki_{High} and Ki_{Low} respectively) of DA and show that 30% ($D2^{High}$) of the sites labeled by [^{3}H]-spiperone are in a high affinity state for DA.

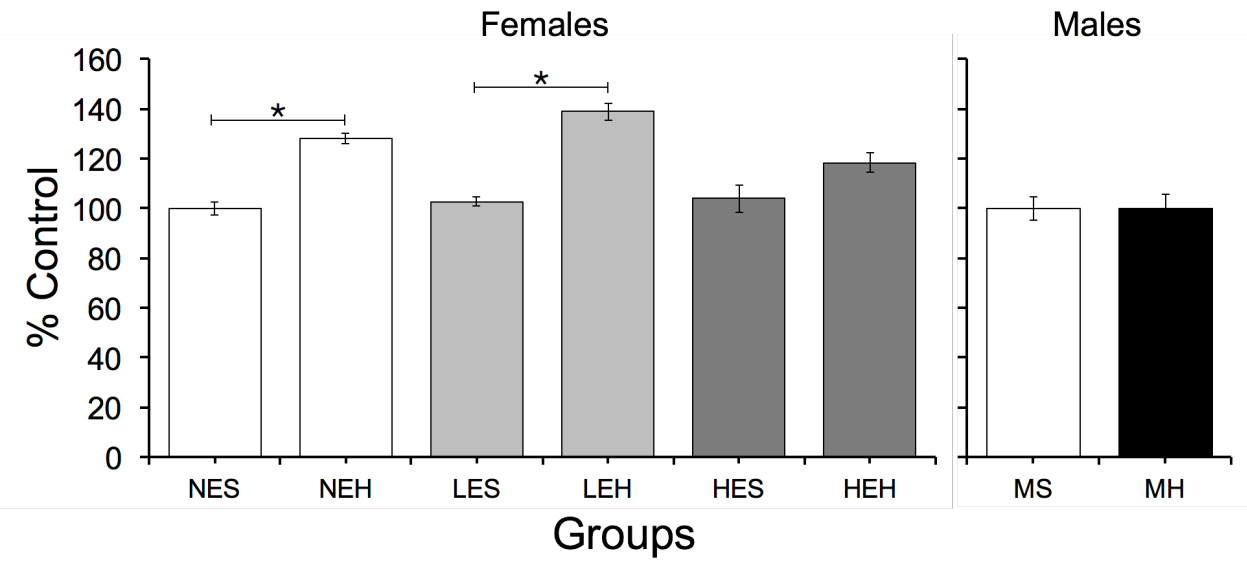

Figure 3. Total binding of D2Rs in the DS in male and female rats receiving no, low, or high E2, in conjunction with saline or HAL, shown as a percentage of controls. Error bars represent standard error of the mean. NES = no E2 + saline; NEH = no E2 + HAL; LES = low E2 + saline; LEH = low E2 + HAL; HES = high E2 + saline; HEH = high E2 + HAL; MS = male saline; MH = male HAL. * = $p < 0.001$.

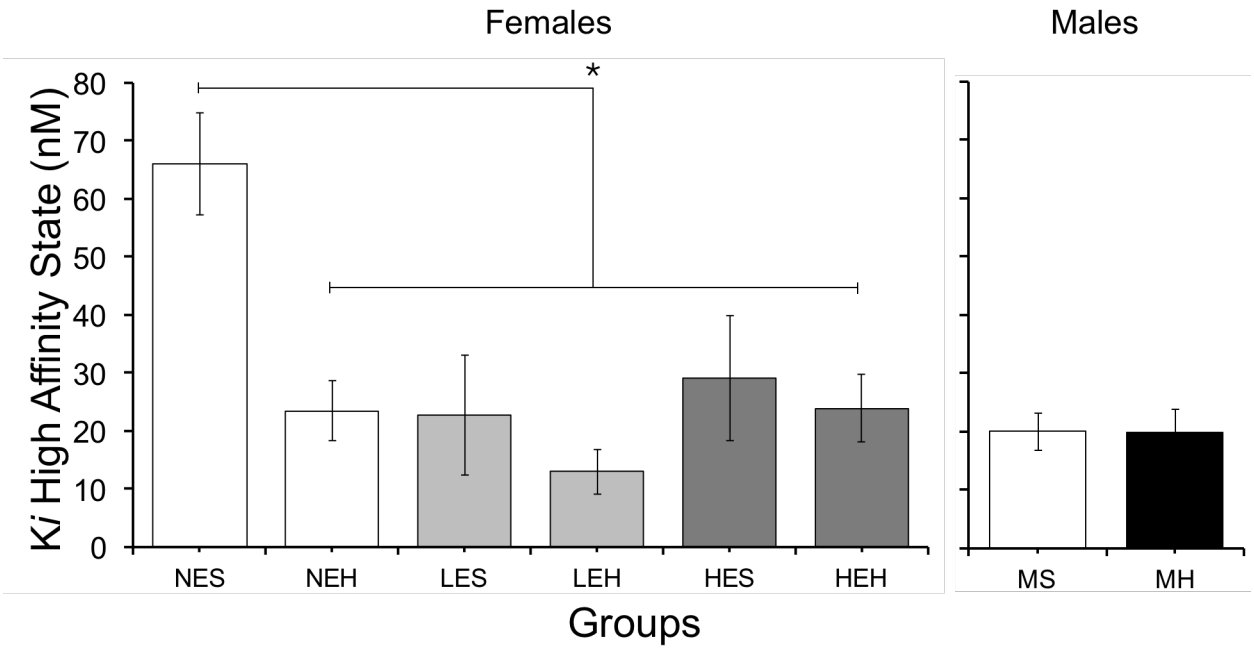

Figure 4. Affinity state of D2Rs in the high affinity state, in nM, in male and female rats receiving no, low, or high E2, in conjunction with saline or HAL. Error bars represent standard error of the mean. NES = no E2 + saline; NEH = no E2 + HAL; LES = low E2 + saline; LEH = low E2 + HAL; HES = high E2 + saline; HEH = high E2 + HAL; MS = male saline; MH = male HAL. * = $p < 0.001$.

the control group that did not receive any drug or hormone treatment. HAL had no effect on the K_i high of D2Rs in male rats ($t_{(15.245)} = 0.031$, $p = 0.976$, $d = 0.014$; *see* Figure 4).

3.2.3 K_i Low of D2Rs

The HAL ($t_{(22)} = 4.700$, $p < 0.001$, $d = 1.781$) and E2 treatment ($t_{(28)} = 2.438$, $p = 0.021$, $d = 1.137$) had significantly higher affinity of $D2^{Low}$ in comparison to the control group (*see* Figure 5). HAL had no effect on the K_i low of D2Rs in male rats ($t_{(11.251)} = 1.501$, $p = 0.161$, $d = 0.707$; *see* Figure 5).

3.2.4 Proportion of High Affinity D2Rs

The HAL ($t_{(22)} = 4.525$, $p < 0.001$, $d = 2.043$) and E2 treatment ($t_{(28)} = 3.159$, $p = 0.004$, $d = 1.536$) significantly decreased the proportion of $D2^{High}$ in comparison to the control group (*see* Figure 6). HAL had no effect on the proportion of $D2^{High}$ in male rats ($t_{(15.997)} = 0.102$, $p = 0.920$, $d = -0.048$; *see* Figure 6).

4. Discussion

Rats were given AMPH administration for 4 consecutive days, then a week of withdrawal, followed by a challenge dose of AMPH. All rats displayed equal or greater locomotor activity on the challenge day, in comparison to the first day of AMPH administration, suggesting that the rats were sensitized to the behavioral effects of AMPH (*see* Figure 1).

It was hypothesized that HAL and E2 treatment may affect the affinity state of D2Rs in an interactive manner. This was the case for total binding, but not for $D2^{High}$ affinity, $D2^{Low}$ affinity, or the proportion of $D2^{High}$. HAL increases total binding of D2Rs in AMPH-sensitized female rats but only when paired with no or low E2 replacement. On the other hand, HAL and E2 treatment increase affinity of $D2^{High}$ and $D2^{Low}$, and decrease the proportion of $D2^{High}$ in AMPH-sensitized female rats. It has been shown that HAL increases the percentage of $D2^{High}$ in AMPH-sensitized male rats, but does not increase D2R binding (Seeman et al., 2005; Seeman, 2009). Here we observed this effect in AMPH-sensitized female rats, but it was dependent upon E2 levels. In AMPH-sensitized OVX female rats HAL increases total D2R binding in those with no or low E2 replacement, but not with high E2 replacement. This may be one reason why following menopause, during the time ovarian estrogen production is at its lowest, women require higher

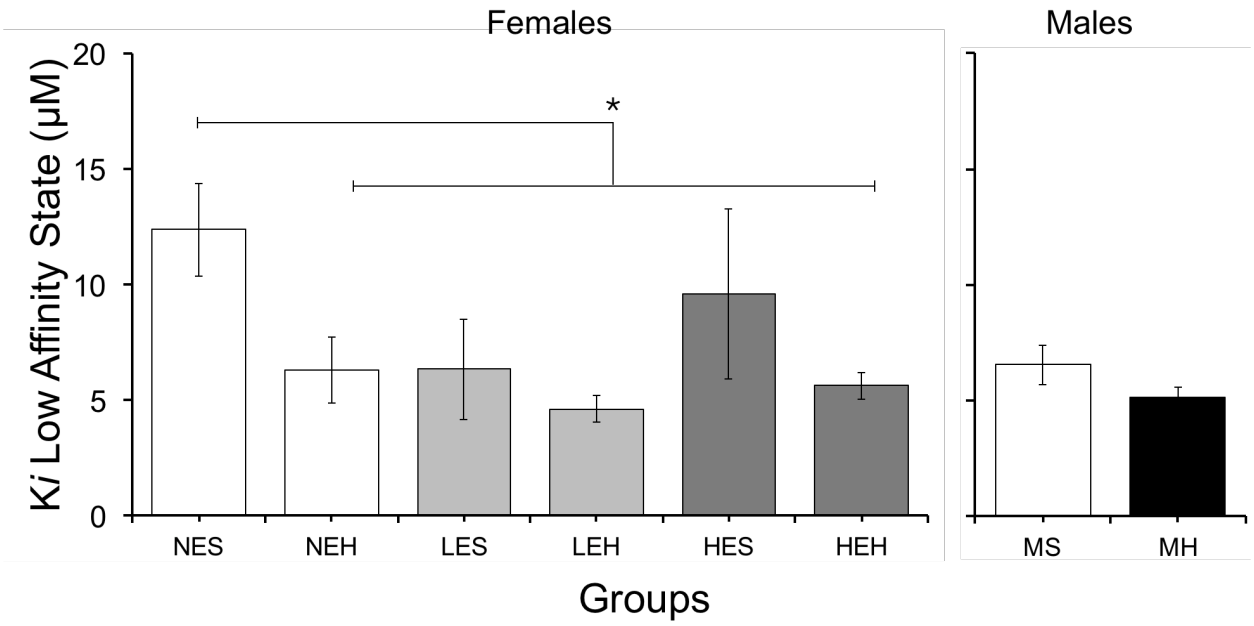

Figure 5. Affinity state of D2Rs in the low affinity state, in μM, in male and female rats receiving no, low, or high E2, in conjunction with saline or HAL. Error bars represent standard error of the mean. NES = no E2 + saline; NEH = no E2 + HAL; LES = low E2 + saline; LEH = low E2 + HAL; HES = high E2 + saline; HEH = high E2 + HAL; MS = male saline; MH = male HAL. * = $p < 0.001$.

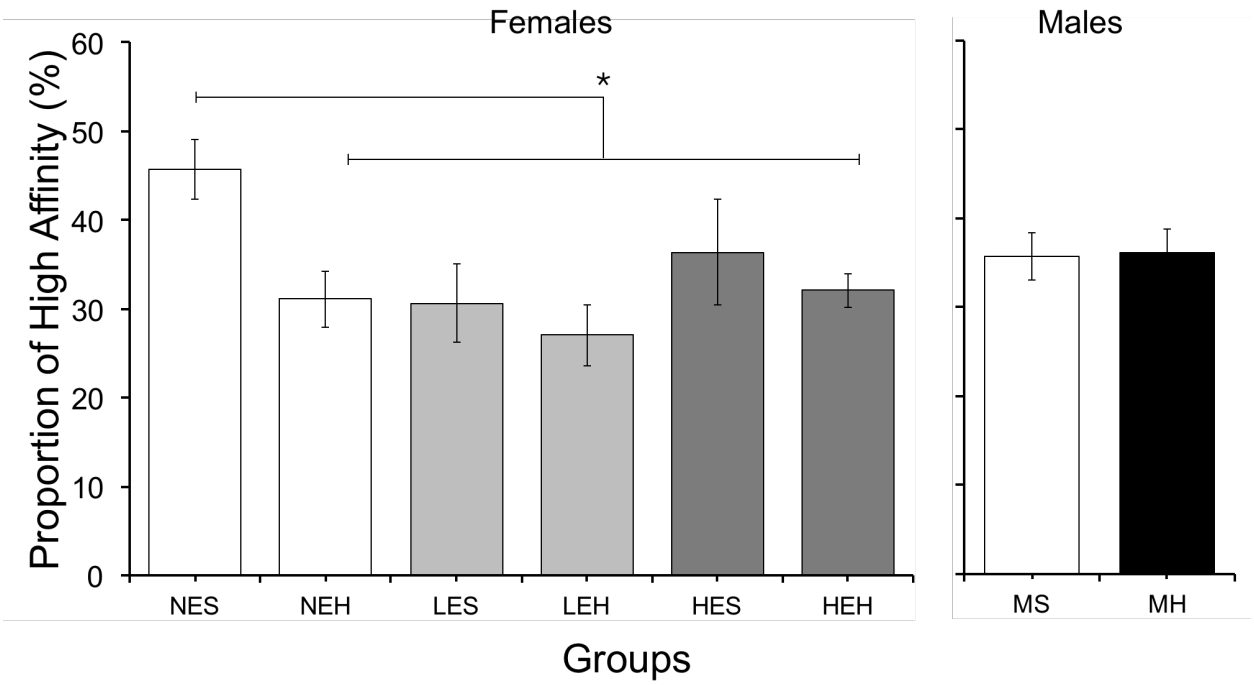

Figure 6. Proportion of D2Rs in the high affinity state, in male and female rats receiving no, low, or high E2, in conjunction with saline or HAL. Error bars represent standard error of the mean. NES = no E2 + saline; NEH = no E2 + HAL; LES = low E2 + saline; LEH = low E2 + HAL; HES = high E2 + saline; HEH = high E2 + HAL; MS = male saline; MH = male HAL. * = $p < 0.001$.

doses of antipsychotics than men of the same age (Seeman, 1983, 1989; Riecher-Rössler and Häfner, 1993). Interestingly, women require lower doses of antipsychotics between the ages of 20 – 40 years, during a time of high ovarian estrogen production, in comparison to older women or men of the same age (Seeman, 1983). In fact, previous studies have shown that estrogen levels have a direct inverse correlation with dose of antipsychotics (Hallonquist et al., 1993; Gattaz et al., 1994; Riecher-Rössler et al., 1994).

HAL and E2 increase the affinity of $D2^{High}$ and $D2^{Low}$ in AMPH-sensitized female rats. This expands upon previous work showing that not only do HAL (Seeman et al., 2005; Seeman, 2009) and E2 (during diestrus; Di Paolo et al., 1988) increase the percentage of $D2^{High}$, but they also increase the affinity of D2Rs in general. That is to say that the overall affinity is increased in both $D2^{High}$ and $D2^{Low}$. This supports previous work showing that E2 increases affinity state. This may be a reason why women generally require lower doses of antipsychotics and have a greater response to the treatment than men (Seeman, 2004). For example, in a study by Tang et al (2007), average daily dose of antipsychotics was significantly lower in women compared to men.

HAL and E2 treatment decrease the proportion of $D2^{High}$ in AMPH-sensitized female rats. This finding supports previous work done by Seeman (2009) showing that HAL (0.25 mg/kg/day, IP) reduces the proportion of $D2^{High}$ by 60% in AMPH-sensitized male rats. It has been suggested that E2 has similar effects on $D2^{High}$ as HAL. For example, during proestrus, when E2 levels are high, there is a decrease in $D2^{High}$ (Di Paolo et al., 1988). Additionally, an acute dose (100 ng) of E2 in OVX rats rapidly converts D2R from $D2^{High}$ into $D2^{Low}$ (Levesque and Di Paolo, 1988). Together these data suggest an additive effect, with both HAL and E2 reducing $D2^{High}$ in the striatum.

HAL had no effect on total binding, affinity or proportion of D2Rs in AMPH-sensitized male rats. The fact that there are sex differences in response to HAL is not surprising. In fact, there are many sex differences that exist in schizophrenia including the age of onset, the course of the disorder, the severity of the symptoms, the treatment, and the side effects of the treatment (Seeman, 1997; *for review, see* Seeman, 2004) amongst others. For example, the age of onset in men is typically between the ages of 15 and 25, whereas the age of onset in women is usually between the ages of 20 and 30, with a second peak occurring around menopause (Sham et al., 1994; *for review, see* Searles et al., 2017). Women have a better course of the disorder than men (Abel et al., 2010). Women exhibit less severe symptomatology than men (Häfner, 2003;

Kulkarni et al., 2008). For example, during the first episode of the disorder, men exhibit greater and more severe negative symptoms (Leung and Chue, 2000). Women are more responsive to treatment (Seeman, 2004), and require lower doses of antipsychotics (Seeman, 1983), at times as low as half the treatment for effective maintenance as men (Melkersson et al., 2001). These sex differences are in part thought to be driven by estrogens, since estrogens have been shown to delay the onset of schizophrenia (Lindamer et al., 1997; Häfner et al., 1998; Bergemann and Riecher-Rossler, 2005; *for review, see* González-Rodriguez et al., 2016). For example, as mentioned before, onset of schizophrenia in women typically occurs during 20 – 30 years of age and at menopause, the same two phases of life when estrogen fluctuation is at its greatest (Prior, 1998).

Shvartsburd et al. (1983) found that individuals with schizophrenia chronically treated with HAL displayed constant high steady-state plasma levels of HAL. Interestingly, Pollock (1997) found that women treated with the same dose of antipsychotics as men had higher steady-state plasma levels of the treatment. This is because women's bodies contain ~25% more adipose tissue than men's bodies, and since most antipsychotics are lipophilic, the distribution of these drugs is greater in women than in men (Beierle et al., 1999). A higher proportion of adipose tissue prolongs the half-life of the antipsychotics, leading to a greater accumulation over time, resulting in higher steady-state plasma levels (Altamura et al., 2003).

Unlike the current findings, Seeman (2009) found that HAL (0.25 mg/kg/day, IP) reduced the AMPH-induced proportion of $D2^{High}$. It is important to note that the route of administration of HAL is different in the two studies. The current study administered a chronic dose of HAL (0.25 mg/kg/day) via minipump in AMPH-sensitized rats, whereas Seeman (2009) administered an intermittent dose of HAL (0.25 mg/kg/day) via intraperitoneal injection. Samaha et al (2008) investigated the effects of chronic (0.5 mg/kg/day; minipump administration) versus intermittent (0.05 mg/kg/day; SC administration) dose treatment of HAL, in AMPH-sensitized male rats. They found that the intermittent dose treatment was more effective than the chronic treatment. Whereas, chronic (0.25 or 0.75 mg/kg/day; minipump administration) treatment of HAL loses its effects over time, leading to treatment failure (Samaha et al., 2007). Given the findings from these studies, it is possible that the null results in male rats are due to the dose and the route of administration of HAL in the current study. It should be noted here that we chose the 0.25 mg/kg/day dose because at a higher dose, the female rats became lethargic and immobile

and did not perform behaviorally (Almey et al., 2013). As such, the male rats were also treated with the same dose so that males and females could be directly compared. Thus, it is not surprising that the current study did not replicate the findings of Seeman (2009) using a much higher dose of HAL in male rats. Future studies should measure the effects of HAL in AMPH-sensitized male rats via this route of administration but at a higher dose of 0.75 mg/kg/day. A 0.75 mg/kg/day dose, but not 0.25 mg/kg/day dose, has an effect on AMPH-induced DA release and locomotor behavior in AMPH-sensitized male rats (Samaha et al., 2007).

HAL and E2 treatment affect not only the D2R binding and affinity, but also behaviors associated with schizophrenia. For example, HAL improves perseveration and reversal learning when AMPH-sensitized OVX rats were treated with E2 replacement (Almey et al., 2017). Additionally, high E2 replacement helps facilitate HAL in restoring latent inhibition in OVX rats (Almey et al., 2013). It is possible that these behavioral changes are driven by the effects of HAL and E2 on the D2R. For example, chronic treatment of HAL, in comparison to acute treatment, reduces AMPH-induced locomotor activity in AMPH-sensitized OVX female rats treated with high E2 (Madularu et al., 2014). Whereas, AMPH-sensitized OVX female rats given saline treatment did not show a reduction in the same behavior. Additionally, AMPH-sensitized OVX female rats given high E2 replacement display reduced AMPH-induced NA DA release when treated with chronic HAL (Madularu et al., 2014). Furthermore, women respond better to treatment for schizophrenia when it is in conjunction with estrogen therapy, in comparison to antipsychotics alone (Kulkarni et al., 1996, 2001; Akhondzadeh et al., 2003; *for review, see* Kulkarni et al., 2012). Thus, the current findings provide further evidence that antipsychotic treatment for schizophrenia may be more effective when it is administered in conjunction with E2.

Chronic HAL affects total binding and affinity of D2Rs in AMPH-sensitized female rats in a manner that is different than its affects in AMPH-sensitized male rats, using a dose that does not affect the affinity or binding of the D2R in male rats. E2 does interact with HAL to affect total binding of D2Rs and affinity for $D2^{High}$ in AMPH-sensitized female rats, but it does not seem to interact with HAL to affect the affinity of $D2^{Low}$ or the proportion of $D2^{High}$. The current findings provide one more explanation as to why we see sex differences in response to antipsychotic treatments. Future studies should measure and compare the effects of chronic and

intermittent HAL (and atypical antipsychotics) in AMPH-sensitized female rats, and at a higher dose in AMPH-sensitized male rats, on the D2R affinity state.

CHAPTER 5:
THE EFFECTS OF 17β-ESTRADIOL AND HALOPERIDOL ON AKT, PHOSPHORYLATED AKT, AND β-ARRESTIN 2 IN AMPHETAMINE-SENSITIZED FEMALE RATS.

Waqqas Shams, Fei Xiang Zhou, Jesse Duchemin, Collin Gagne, and Wayne Brake

Preface:

The findings from Chapter 4 showed that E2 and HAL increase the affinity state of both $D2^{High}$ and $D2^{Low}$, while decreasing the proportion of D2R in the high affinity state. These results help identify one potential mechanism through which E2 affects DA transmission. As such, we were interested to know if this mechanism involves the second messenger cascade activated by D2Rs. Therefore, in Chapter 5, we examined the effects of E2 and HAL on second messenger cascade proteins including Akt, phosphorylated Akt, and β-arrestin 2 activated by D2Rs in AMPH-sensitized female rats. These proteins were semi-quantified within the PFC, the NA, the DS, and the SN using the Western Blotting technique.

Abstract:

Sex differences in onset and symptom manifestation of schizophrenia have led researchers to question whether sex hormones play a role in the neurobiology of this disorder. Previous literature has shown that individuals with schizophrenia have lower levels of phosphorylated Akt (pAkt), a protein implicated in cell survival. E2 has been shown to rapidly stimulate phosphorylation of Akt. Thirty-six female rats were OVX and received either no, low, or high E2 replacement. All rats were AMPH-sensitized and were administered either chronic HAL or saline. Akt, pAkt, and β-arrestin levels were then examined in the PFC, the NA, the DS, and the SN. High E2 treatment reduced immunoreactivity of pAkt (Thr308) in the high E2 saline group in comparison to the no E2 saline group in the DS, while HAL treatment increased immunoreactivity of pAkt (Ser473) in comparison to the saline treatment in the PFC. No significant differences were observed in this study, suggesting that E2 and HAL do not alter the levels of Akt, pAkt, and β-arrestin in AMPH-sensitized female rats. Future studies should investigate the effects of E2 and HAL on Akt, pAkt, and β-arrestin in both AMPH and non-AMPH-sensitized rats.

1. Introduction

Schizophrenia is a chronic neurodevelopmental disorder affecting approximately 1% of the population, with roughly similar rate of prevalence in men and women (Konopaske and Lewis, 2007). The original version of the DA hypothesis of schizophrenia proposes that this disorder is caused by hyperactive DA transmission (Seeman, 1987). DA is one of the major neurotransmitters targeted by the treatments for schizophrenia. Important for many functions including cognition, motor control, motivation, learning, and reward, DA is released within brain regions in the basal ganglia including the DS and VS, the PFC, and the SN (Bannon et al., 2012).

The majority of the treatments currently available for schizophrenia target the D2R. D2Rs have two affinity states, a high affinity state, where they are functional, and a low affinity state, where they are functionally inert (Seeman, 1982; Wreggett and Seeman, 1984). When activated, D2Rs can utilize a number of second messenger cascades including inhibition of adenylyl cyclase, preventing signal amplification (Neve et al., 2004; Bockaert, 2009), and phosphorylation of GRK. GRK phosphorylation in turn activates β-arrestin, a scaffolding protein critical for G-protein coupled receptor desensitization and trafficking (Beaulieu et al., 2007). β-arrestin recruitment in turn results in a formation of a signaling complex between β-arrestin, PP2A (a protein that is important for regulation of the cell cycle, signal transduction, cell differentiation, and transformation), and protein kinase B, also referred to as Akt. The formation of this signaling complex results in deactivation of Akt. Akt is a kinase that is important for a number of functions including inhibiting cell death (apoptosis) and promoting cell survival (proliferation) amongst other functions (Beaulieu et al., 2007).

It could be speculated that the affinity state of D2R is directly related to levels of β-arrestin and pAkt. That is, more D2Rs in the high affinity state could result in a higher level of β-arrestin and lower levels of pAkt (i.e. activated Akt). Lower levels of Akt have been observed in the frontal cortex and hippocampus of individuals with schizophrenia (Emamian et al., 2004). Some antipsychotics have been shown to affect phosphorylation of Akt (Masri et al., 2008; *for review, see* Beaulieu, 2012). For example, acute and chronic administration of HAL has been shown to increase levels of Akt in mice (Emamian et al., 2004). Interestingly, estrogens have been shown to rapidly stimulate phosphorylation of Akt in mouse and rat hippocampus (McEwen et al., 2001; Akama and McEwen, 2003; Znamensky et al., 2003; Yildirim et al., 2011). In addition, E2 has been shown to enhance DA release (Becker and Cha, 1989; Becker,

1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999; Shams et al., 2016) and affect D2Rs (Di Paolo et al., 1988; Levesque and Di Paolo, 1988). For example, direct infusion of E2 into the DS increases AMPH-induced striatal DA release (Shams et al., 2016), while an acute dose (100 ng) of E2 causes D2Rs to rapidly switch from the high affinity state to the low affinity state (Levesque and Di Paolo, 1988). Thus, it is possible that estrogens may be interacting with antipsychotics like HAL via this mechanism and this may explain how estrogens facilitate HAL's effects in treating schizophrenia.

It is thought that the positive symptoms of schizophrenia are a consequence of excessive DA transmission in the NA, while the negative and cognitive symptoms are a consequence of DA hypoactivity in the PFC (Neve, 2010). The DS is a brain region commonly targeted in studies on schizophrenia and its treatments (*for review, see* Seeman, 2006). Given that the SN sends DAergic projections to the DS, the four brain regions examined in the current study were: the PFC, the DS, the NA, and the SN.

Research in our lab has used AMPH-sensitization as a rat model of some of neurochemical and behavioral changes seen in schizophrenia. Recently, we found that a 0.25 mg/kg/day dose of HAL in AMPH-sensitized OVX rats with low E2 replacement improves perseveration and reversal learning. In addition, a lower dose of 0.13 mg/kg/day in AMPH-sensitized OVX rats with high E2 replacement also improves reversal learning (Almey et al., 2017). High E2 replacement in OVX rats also facilitates the effects of acute 0.5 mg/kg HAL treatment on latent inhibition (Almey et al., 2013). OVX rats treated with chronic HAL and high E2 replacement display enhanced blood-oxygen-level-dependent activity in the hippocampal formation, habenula, amygdala, and hypothalamus, amongst other regions of interest associated with schizophrenia (Madularu et al., 2016). Lastly, AMPH-sensitized OVX rats treated with chronic HAL and high E2 replacement display reduced locomotor activity and AMPH-induced NA DA release (Madularu et al., 2014).

Based on these findings, it was hypothesized that HAL and E2 treatment may affect D2R affinity state, and in turn affect levels of Akt, pAkt and β-arrestin in the PFC, the DS, the NA, and/or the SN. AMPH-sensitized OVX female rats underwent HAL or saline treatment, receiving no, low, or high E2 replacement. It was hypothesized that HAL treatment, E2 replacement, individually or their combination may have interactive effects on Akt, pAkt or β-arrestin 2.

2. Methods

2.1 Animals

Female Long-Evans rats (Charles River Laboratories, St. Constant, QC, Canada) weighing 250-350 g were single housed at 21°C in polypropylene shoebox cages with beta-chip bedding. A 12:12 h reverse light/dark cycle was used with lights off at 9:00 AM. Rats had access to *ad libitum* Purina rat chow and water. Handling, testing and surgical procedures were performed during the dark phase of the diurnal cycle. Protocols were previously approved by Concordia University's animal research ethics committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Ovariectomy

Thirty-six rats were anesthetized using Isoflurane (4% for induction; 2% for maintenance; Inhalation Anaesthetic, Richmond Hill, ON, Canada) and bilaterally OVX through a dorsal incision (1 cm). For post-surgical care all rats were administered the anti-inflammatory drug, Anafen (0.1 mL/rat, SC; MERIAL Canada Inc., Morgan Baie d'Urfe, QC, Canada), the antibiotic Penicilin G (0.2mL/rat, intramuscular; CDMV, St. Hyacinthe, QC, Canada) and antibiotic ointment (By Pharmaceuticals Inc., Brampton, ON, Canada) was applied to the incision. Following ovariectomy, rats were given one week to recover in their home cages.

2.3 AMPH Sensitization

All rats were habituated to the locomotor activity chambers for 2 hr/day for two consecutive days along with IP injection of saline prior to the induction phase of AMPH-sensitization. *D*-amphetamine sulphate (1 mg/kg or 0.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline and repeatedly administered via intraperitoneal injection (IP) to induce AMPH sensitization. Rats were administered 1 mg/kg AMPH daily for four consecutive days while locomotor activity was measured (Robinson, 1984; Robinson and Becker, 1986). Spontaneous locomotor behavior during AMPH sensitization was assessed 30 minutes prior and 90 minutes following AMPH administration, using locomotor activity chambers (42 cm x 25 cm x 17 cm). The behavior was monitored for a period of 120 min by recording interruptions of photobeams of two pairs of photocells at 14 cm increments, located 2.5 cm above the grid floor that were interfaced to a MED control system with MED-PC software (MED Associates Inc., ENV-013). The monitoring session was divided into pre-injection (30 min) and post-injection (90 min) components, during which MED-PC software

recorded total time spent moving. All rats were tested throughout the experiment in the same respective activity chamber at the same time of day. After a seven-day withdrawal period, rats were administered an initial AMPH challenge (0.5 mg/kg IP; expression) to determine whether they exhibited sensitization to the locomotor stimulating effects of AMPH. AMPH-sensitization was considered to have developed if rats exhibited comparable or higher levels of locomotor activity in response to the challenge dose of AMPH (*see* Figure 1) in comparison to the activity of day 1, where they were injected with a higher dose of AMPH. This protocol in our lab has previously been shown to induce AMPH sensitization of locomotor activity and accumbal DA release (Madularu et al., 2014).

2.4 E2 Capsule Implantation

Following AMPH challenge, rats were implanted with a silastic tube (1 cm long; 1.96 mm OD, 1.47 mm ID) containing either 5% E2 (Sigma Chemical Co., St. Louis, MO, USA) in cholesterol (Sigma; $n = 24$) or cholesterol only ($n = 12$) in the nape region and divided into three hormone condition groups; no E2 ($n = 12$), low E2 ($n = 12$), and high E2 ($n = 12$).

Low E2 was administered via silastic capsule containing E2 in cholesterol. These capsules have previously shown to mimic low physiological serum concentrations of ~20 – 25 pg/ml E2 consistent with estrus (Almey et al., 2013). In addition to the capsules, rats in the high E2 group received injections of E2 in sesame oil once every two days (10 µg/kg, SC), starting after E2 capsule implant and before the minipump implantation. We have shown that the combination of the E2 capsule and SC E2 injection achieves an average plasma level of ~90 pg/ml across 12 hours (Almey et al., 2013), similar to average plasma levels of E2 consistent with the proestrus phase of the estrous cycle (Butcher et al., 1974). Rats in the no E2 and low E2 groups received sesame oil injections.

2.5 HAL Treatment

Rats in each of the three hormone conditions were randomly divided into 2 drug treatment groups, HAL or saline. The HAL (0.25 mg/kg/day; Sandoz Canada Inc., QC, Canada) group ($n = 18$) was implanted with Alzet osmotic minipumps (model: 20002; Durect, Cupertino, CA, USA) that released HAL at a rate of 0.5 µL/h for 14 days, while the saline group ($n = 18$) was implanted with the same minipumps that released saline in a similar manner. Delivery of

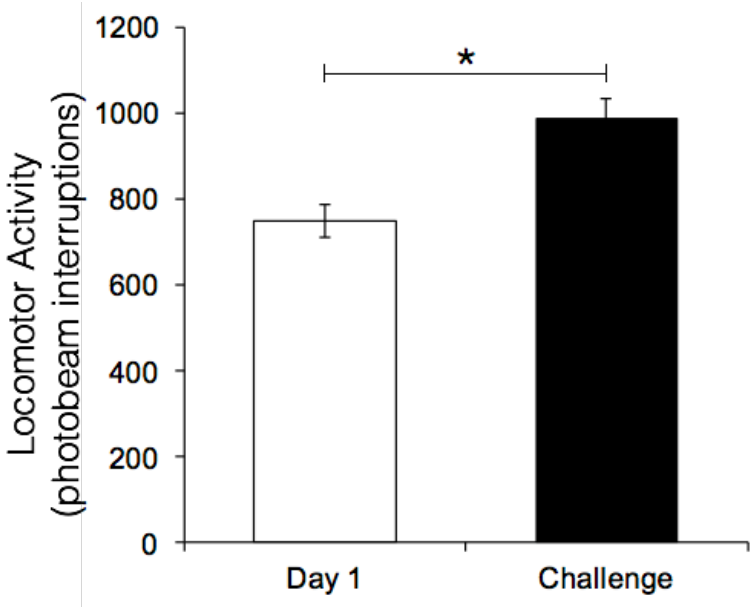

Figure 1. Comparison of mean (± SEM) locomotor activity observed on day 1 of AMPH injections (1.0 mg/kg IP) to the challenge day injection (0.5 mg/kg IP) in rats following AMPH withdrawal. Greater locomotor activity on challenge day suggests that behavioral sensitization has occurred. $p < 0.001$.

0.25 mg/kg/day of HAL via minipump has been shown to produce a steady state D2 receptor occupancy in rats, closely approximating the pharmacokinetic profile of effective drug doses observed in humans (Samaha et al., 2007, 2008).

On the 15th day of minipump implantation, rats were decapitated and their brains were removed and flash frozen with 2-methylbutane (Isopentane) in dry ice and stored at -80 °C (*see* Figure 2 for complete experimental timeline).

2.6 Western Blotting

Tissue Extraction. Brains were sliced on a cryostat along the coronal plane and specific slices for regions including the PFC, NA, DS, and SN were collected. Brain slices of 400 µm were obtained and bilateral tissue punches of the PFC, NA, DS, and SN were collected using a micron tissue biopsy punch in aliquots and stored at -80 °C for further analyses.

Protein Quantification. Tissue punches were kept frozen at all times until homogenization. The protein in each sample was quantified through the addition of 75 µl (PFC), 75 µl (NA), 100 µl (DS), or 40 µl (SN) of homogenization lysate buffer (10 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 1x tablet Protease inhibitor cocktail tablet, 0.1 mL of Phosphatase Inhibitor Cocktail 2 and 3). Samples were sonicated 3x each for 10 – 15 sec at a time, with samples kept on ice in between sonication. Next, samples were frozen and heated three times using liquid nitrogen and a heat block set at 37 °C, respectively. Homogenates were then centrifuged at 7,000 RCF for 30 min at 4 °C. The protein content of the supernatant was quantified using a Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Pierce Protein Research Products, Rockford, IL, USA). The supernatant was then stored at -80 °C until ready for Western blot analyses.

Polyacrylamide Gel Electrophoresis. Samples consisting of 20 µg of protein, in a final volume of 25 µl were separated on a 12% homemade SDS-polyacrylamide gel (30% Acrylamide mix, 1.5 M Tris-HCl, pH 8.8, 10% SDS, 10% APS, 8 µl N, N, N', N'-Tetramethylethylenediamine) for 10 – 15 min at 150 V, followed by 150 min at 120 V. Included in each gel was 2 µl of Precision Plus Protein Dual Color Standards (Bio-Rad; cat. no. 161-0394) for band weight comparison. Proteins were transferred onto a nitrocellulose membrane (0.2 µM; Bio-Rad, cat. no. 162-0112) using constant current at 100 V for 60 min.

A SNAP i.d. 2.0 protein detection system (Millipore, MA, USA) was used to incubate membranes in blocking solution, primary and secondary antibodies, and washes in between

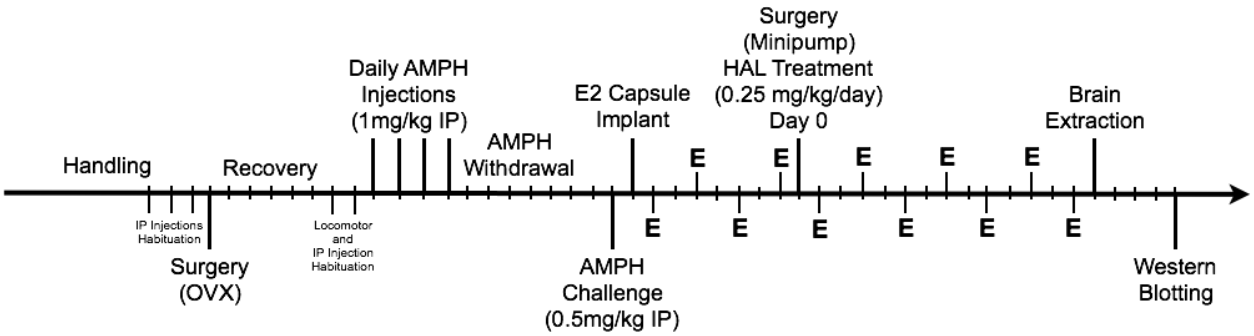

Figure 2. Schematic of the experimental timeline. After handling, rats underwent ovariectomy surgery. Following surgery, rats received four daily injection of AMPH (1.0 mg/kg IP). One week after the last AMPH injection, rats received an AMPH challenge injection (0.5 mg/kg IP). Next, rats were implanted subcutaneously with either E2 or cholesterol capsules, followed by either HAL or saline containing delivery minipumps, a week after. Rats were treated with either E2 or oil injections every other day. 14 days after minipump implant, brains were extracted and frozen for western blotting. OVX = ovariectomized; AMPH = amphetamine; E2 = 17β-estradiol; HAL = haloperidol; E = 17β-estradiol or oil injection.

incubations. Transferred membranes were placed inside a blot holder (Millipore, MA, USA, cat. no. 41623-1-3) and were blocked with 1% bovine serum albumin (BSA) in TBS-Tween-20 (TBST) solution and incubated for 60 min with primary antibodies at room temperature. Primary antibodies recognize protein for total Akt (1.5:500; Cell Signaling Technology; cat. no. 4691), phosphorylated Akt Ser473 (1:500; Cell Signaling Technology; cat. no. 4060), phosphorylated Akt Thr308 (1:500; Cell Signaling Technology; cat. no. 13038), β-Arrestin 2 (1:250; Cell Signaling Technology; cat. no. 3857), and β-Actin (0.1:5000; Millipore, MA, USA; cat. no. MAB1501) in 1% BSA-TBST solution.

Following incubation, membranes were washed four times in TBST (0.1% Tween) and incubated for 10 min at room temperature with secondary anti-rabbit IgG, HRP-linked antibody (1:5000; Cell Signaling Technology; cat. no. 7074), goat anti-mouse IgG, peroxidase conjugated, H+L antibody (2:5000; Millipore, MA, USA; cat. no. AP124P), and a ladder secondary (Precision Protein StrepTactin-HRP Conjugate; 0.83:5000; Bio-Rad; cat. no. 161-0381) in a 0.5% milk-TBST solution. Membranes were again washed four times in TBST (0.1% Tween) and covered with an enhanced chemiluminescence substrate (Western Lightning Plus-ECL, PerkinElmer Inc., MA, USA) for 5 min, before being developed using an Imager (Amersham Imager 600). Images were taken at 1 min and used for analysis.

Protein densities were semi-quantified using the Gel Analysis method within ImageStudioLite software. Protein levels were standardized by dividing the densities of the Akt, pAkt (Ser473), pAkt (Thr308), and β-Arrestin 2 bands by the density of the β-Actin band for each sample.

2.7 Statistical Analyses

The effects of hormone or HAL treatment were analyzed using a two-way ANOVA with hormone group and HAL treatment as a between-factor for each brain region and each primary antibody separately. When appropriate Tukey's HSD post-hoc analyses were conducted. Eta squared or partial eta squared were also conducted to quantify the magnitude of the effect between groups.

3. Results

Two-way ANOVA. Hormone and drug treatment for each brain region and each protein were compared separately. An interaction between drug treatment and hormone treatment ($F_{(2, 36)}$

= 5.099, $p = 0.012$, $\eta_p^2 = 0.254$) was observed for phosphorylated Akt (Thr308) in the DS (*see* Figure 3). Tukey's HSD revealed that high E2 treatment reduced immunoreactivity of phosphorylated Akt (Thr308) in the HES group in comparison to the NES group ($p = 0.046$).

A main effect of drug ($F_{(1, 36)} = 4.307$, $p = 0.047$, $\eta_p^2 = 0.126$) was observed for phosphorylated Akt (Ser473) in the PFC (*see* Figure 4). These data show that HAL treatment increased immunoreactivity of phosphorylated Akt (Ser473) in comparison to saline treatment. No other significant differences were observed (*see* Figures 5 and 6).

t-test. A dependent samples *t*-test was conducted to compare day 1 locomotor activity to challenge day locomotor activity to see if rats were AMPH sensitized. Dependent samples *t*-test revealed that in fact the challenge day locomotor activity was significantly greater than day 1 locomotor activity ($t_{(47)} = 7.329$, $p < 0.001$, $d = 2.604$; *see* Figure 1). These results show that the rats were AMPH sensitized.

4. Discussion

Rats were given AMPH administration for 4 consecutive days, then a week of withdrawal, followed by a challenge dose of AMPH. All rats displayed greater locomotor activity on the challenge day, in comparison to first day of AMPH administration, suggesting that the rats were sensitized to the behavioral effects of AMPH (*see* Figure 1).

It was hypothesized that HAL and E2 treatment, individually or in an interactive manner, may alter Akt, pAkt, and β-arrestin 2 levels through their effects on D2R affinity state. However, this was not the case in the current study. Although high E2 treatment reduced immunoreactivity of pAkt (Thr308) in the HES group in comparison to the NES group in the DS, while HAL treatment increased immunoreactivity of pAkt (Ser473) in comparison to the saline treatment in the PFC, no other significant differences were observed.

There are several potential reasons why the current study failed to find differences among the treatments. For example, the current study used AMPH-sensitization as a model of some of the symptoms of schizophrenia to investigate HAL and E2's effects on Akt, pAkt, and β-arrestin 2. AMPH-sensitization is one of the many animal models used to study the symptoms of schizophrenia. In fact, there are four different categories of animal models used to study symptoms of schizophrenia including, neurodevelopmental, lesion, genetic, and pharmacological

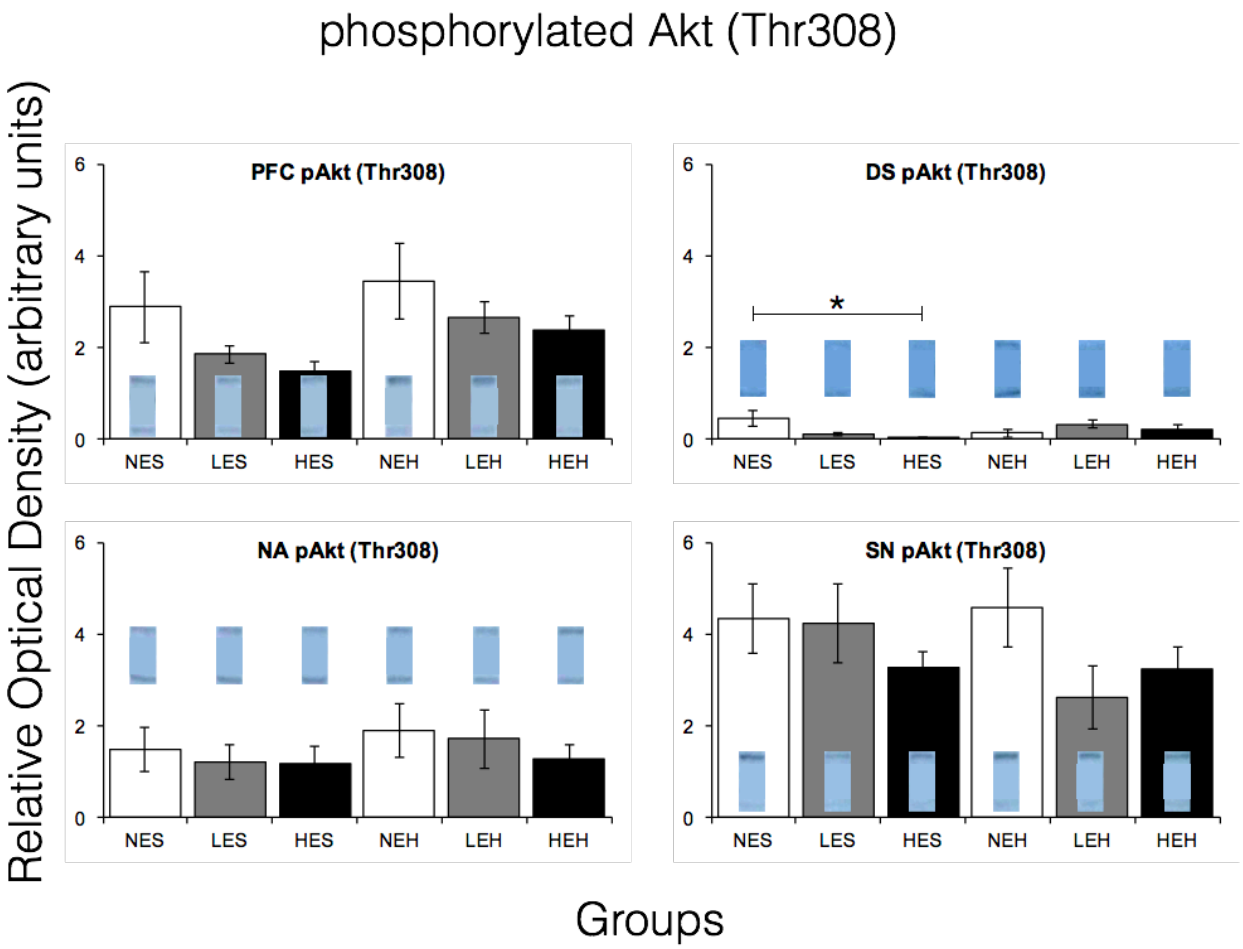

Figure 3. Proportion of relative optical density of phosphorylated Akt (Thr308). Upper bands represent targeted protein for each group, while the lower bands represent the control β-actin. NES = no E2 + saline; LES = low E2 + saline; HES = high E2 + saline; NEH = no E2 + HAL; LEH = low E2 + HAL; HEH = high E2 + HAL. $p = 0.012$.

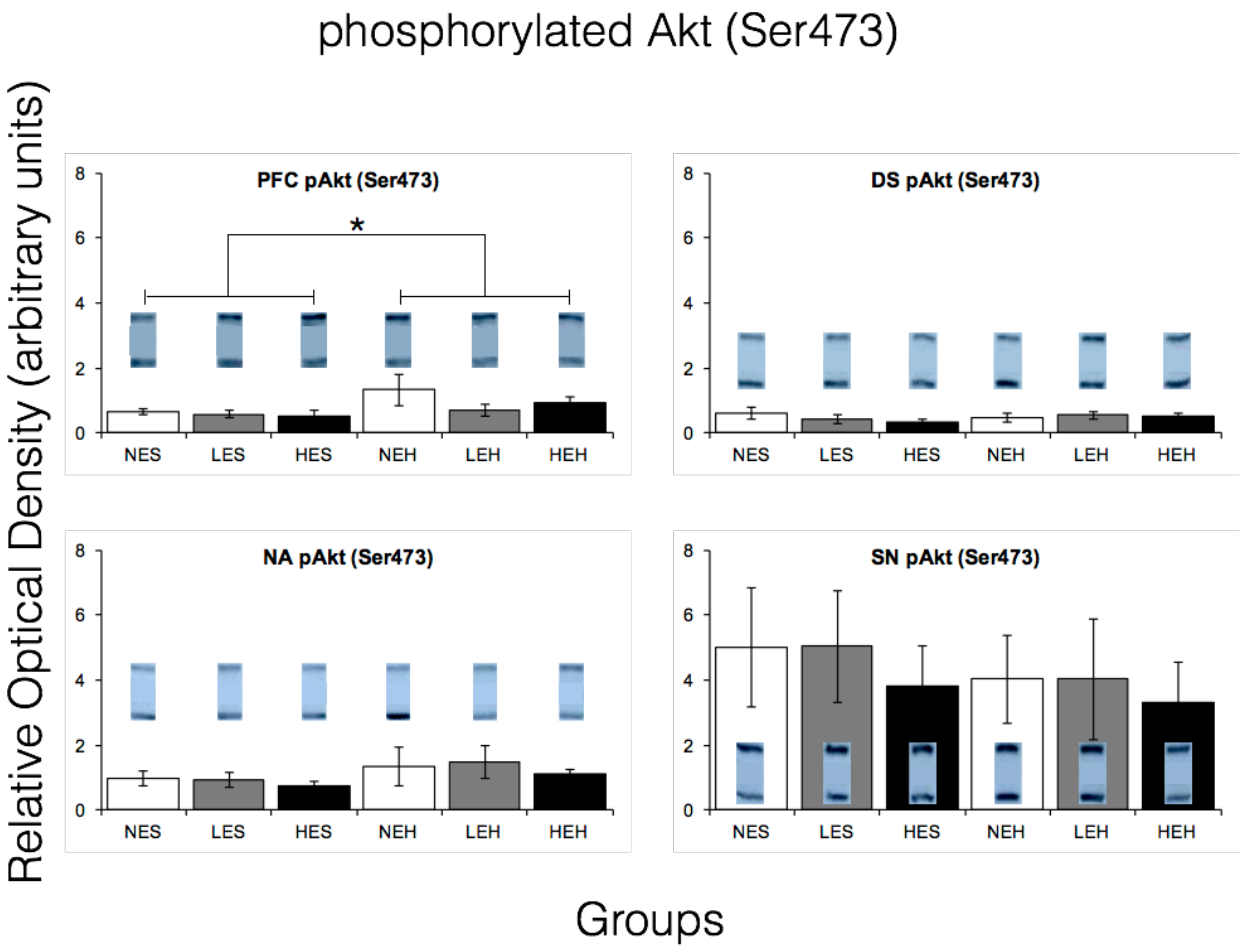

Figure 4. Proportion of relative optical density of phosphorylated Akt (Ser473). Upper bands represent targeted protein for each group, while the lower bands represent the control β-actin. NES = no E2 + saline; LES = low E2 + saline; HES = high E2 + saline; NEH = no E2 + HAL; LEH = low E2 + HAL; HEH = high E2 + HAL. $p = 0.047$.

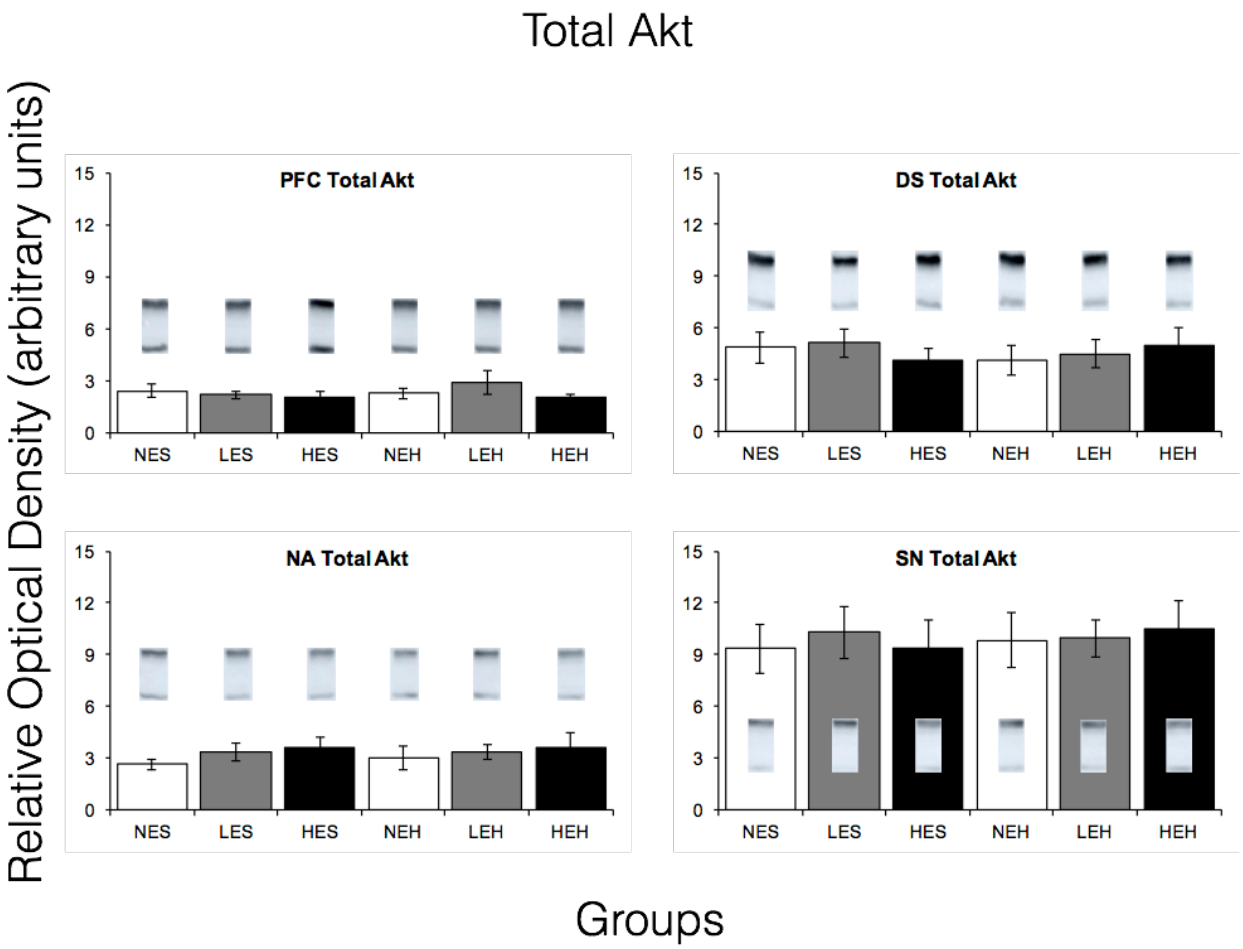

Figure 5. Proportion of relative optical density of total Akt. Upper bands represent targeted protein for each group, while the lower bands represent the control β-actin. NES = no E2 + saline; LES = low E2 + saline; HES = high E2 + saline; NEH = no E2 + HAL; LEH = low E2 + HAL; HEH = high E2 + HAL.

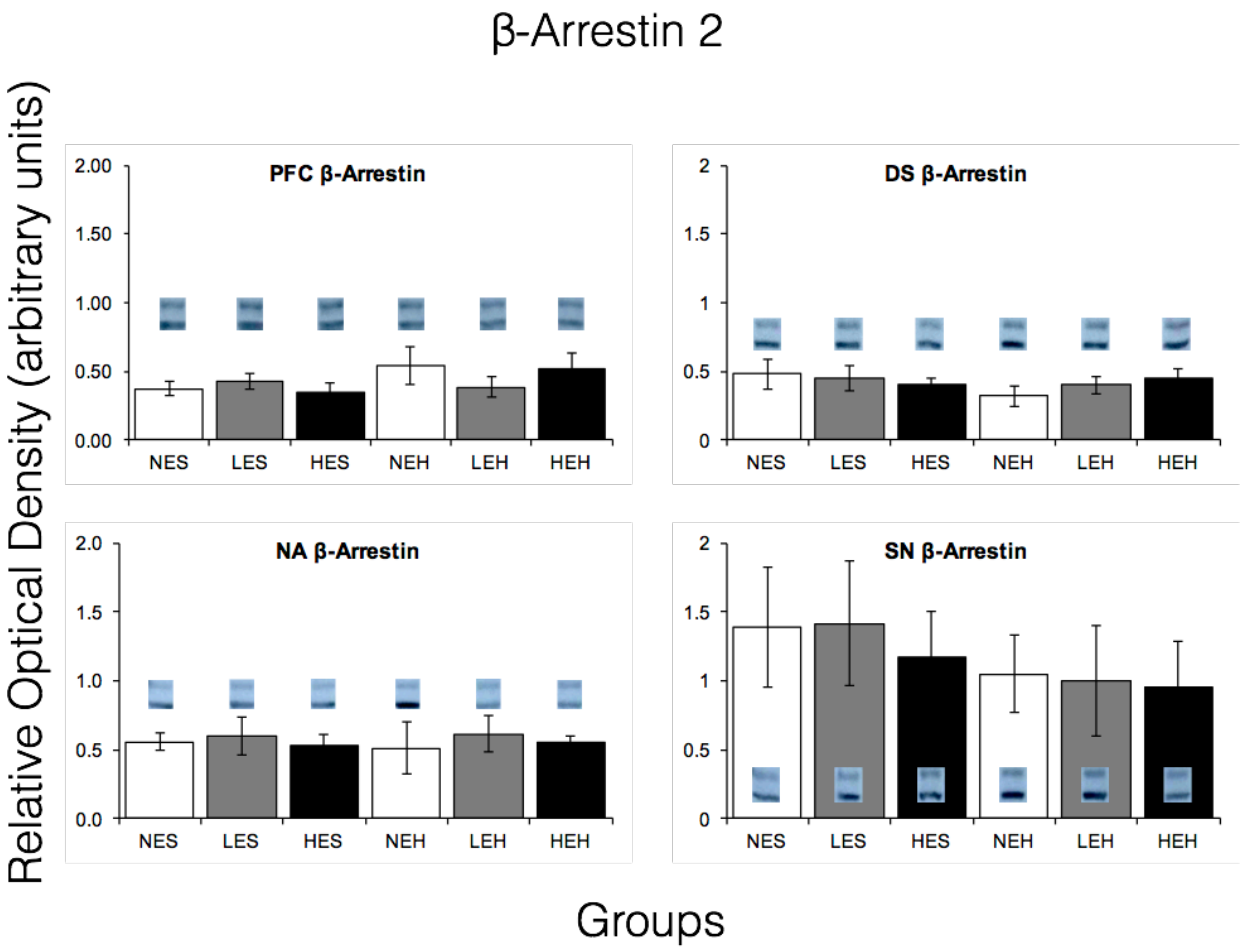

Figure 6. Proportion of relative optical density of β-arrestin 2. Upper bands represent targeted protein for each group, while the lower bands represent the control β-actin. NES = no E2 + saline; LES = low E2 + saline; HES = high E2 + saline; NEH = no E2 + HAL; LEH = low E2 + HAL; HEH = high E2 + HAL.

(Jones et al., 2011). AMPH-sensitization falls under the pharmacological therapy; it was used given the fact that long-term use of AMPH or other psychostimulants have been shown to induce psychosis in humans (Lieberman et al., 1987; Curran et al., 2004; *for review, see* Featherstone et al., 2007). AMPH psychosis is clinically indistinguishable from the positive symptoms of schizophrenia. As such, researchers have used repeated administration of AMPH in animals as a model of some of the symptoms of schizophrenia. Interestingly, it has been shown that AMPH administration to non-transgenic mice (Emamian et al., 2004) or to wild-type mice dephosphorylates Akt (Beaulieu et al., 2005). Perhaps these findings could account for the null results due to the possibility that AMPH-sensitization in the current study may have altered Akt production. Although, estrogens have been shown to rapidly stimulate phosphorylation of Akt (McEwen et al., 2001; Akama and McEwen, 2003; Znamensky et al., 2003; Yildirim et al., 2011), while HAL treatment increases levels of Akt (Emamian et al., 2004), the interactive effects of the two may not have been sufficient against the effects of AMPH to produce significant differences in the current study.

There is extensive research showing that E2 enhances DA release (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999; Shams et al., 2016) and affects D2Rs (Di Paolo et al., 1988; Levesque and Di Paolo, 1988). Estrogens can alter the affinity state of the D2R. For example, E2 increases the affinity of D2R in the high and low affinity state (Shams et al., *submitted*). Additionally, rats have the most D2Rs in the high affinity state during diestrus 2 and the most D2Rs in the low affinity state during estrus and proestrus (Di Paolo et al., 1988). Given these findings, it can be speculated that E2 enhances DA release through its effects on the affinity state of the D2R. As such, it was hypothesized that E2 individually, or in conjunction with HAL may affect D2R-related second messenger proteins. However, it has been shown that elevated DA levels in the striatum of DA transporter-knockout mice reduces Akt phosphorylation (Beaulieu et al., 2004, 2006). Although estrogens have been shown to stimulate phosphorylation of Akt in the hippocampus, it is possible that increased DA levels in the DS in response to E2 administration reduce Akt phosphorylation, resulting in null findings. Thus, investigating the effects of E2 on the targeted proteins in the DS may not have been sufficient to show significant differences.

The current study examined the effects of E2 and HAL in the PFC, DS, NA, and SN; brain regions associated with schizophrenia. However, these areas are not the only brain regions

involved in schizophrenia. Regions including the VTA and the hippocampus are also implicated in schizophrenia (*for review, see* Goldman and Mitchell, 2004). For example, estrogens have been shown to stimulate Akt phosphorylation in the rat hippocampus (Akama and McEwen, 2003), while the VTA projects DAergic terminals to the PFC and the NA (Neve, 2010). Thus, it may be beneficial for future studies to look at the effects of E2 and HAL on the targeted proteins in the VTA and the hippocampus, given that the areas examined did not show any differences.

β-actin is commonly used as a control protein in the Western blotting technique. This is because β-actin is assumed to be present to the same degree in every neuron throughout the brain (*for review, see* Cingolani and Goda, 2008). The current study used β-actin as the control to measure protein in comparison to the targeted proteins. β-actin is important for cell motility, cell morphogenesis, cell division, and synaptogenesis, and is most abundant at dendritic spines (*for review, see* Cingolani and Goda, 2008). Interestingly, E2 also has been shown to influence synaptogenesis. For example, estrogens have been shown to induce synapse formation (McEwen et al., 2001). Specifically, synapses in the hippocampus fluctuate across the rat estrous cycle, with most synaptogenesis occurring during the highest concentration of estrogen in the brain (*for review, see* Znamensky et al., 2003). Thus, it is possible that estrogens' effects on synaptogenesis can also alter the density of β-actin. Therefore, the null results in the current study may have been due to the effects of estrogens on synaptogenesis, and thus on the β-actin control. Future studies should consider using a different control protein when investigating the effects of E2 and HAL on Akt, pAkt, and β-arrestin 2.

As previously discussed, the affinity state of the D2R may be directly related to β-arrestin and pAkt such that more D2Rs in the high affinity state result in higher levels of β-arrestin and lower levels of pAkt. There are two subtypes of β-arrestin, 1 and 2, and both have been implicated in the second messenger cascade that results in deactivation of pAkt. However, only β-arrestin 2 has been shown to directly influence the formation of the signaling complex that inactivates pAkt (Beaulieu et al., 2007). Interestingly, β-arrestin 2 does not always inactivate pAkt. Activated D2R utilizes the second messenger cascade that phosphorylates GRK. GRK in turn recruits β-arrestins that can also take part in a different process from the one that inactivates pAkt (Beaulieu et al., 2007). Beaulieu and colleagues (2005) found that β-arrestin 2 knockout mice displayed suppressed locomotor activity in response to AMPH. Additionally, AMPH administration reduces pAkt levels (Beaulieu et al., 2005). Given these findings, it is evident that

the AMPH-sensitized rats in the current experiment had sufficient β-arrestin 2 present. Additionally, since AMPH reduces pAkt, it is possible that this reduction in pAkt in turn resulted in an increase in β-arrestin 2 density. As such, the lack of difference in the targeted protein density may be due to the reduction in pAkt, a floor effect, or an increase in β-arrestin 2, a ceiling effect, through AMPH administration. Given that the current study did not include a non-AMPH-sensitized group, the effects of AMPH-sensitization on the densities of the targeted proteins could not be measured or addressed. Future studies should investigate the effects of E2 and HAL on Akt, pAkt, and β-arrestin 2 in both AMPH and non-AMPH-sensitized rats.

CHAPTER 6:
GENERAL DISCUSSION

THE EFFECTS OF 17β-ESTRADIOL ON STRIATAL DOPAMINE TRANSMISSION: ITS IMPLICATIONS FOR ANTIPSYCHOTICS.

The goal of this thesis was to address the relationship between E2 and DA by investigating the effects of E2 on DA release, and the mechanisms through which E2 affect DA transmission. As such, the effects of E2 on tonic and phasic DA release were addressed in Chapters Two and Three. The mechanisms through which E2 and HAL affect DA transmission were investigated in Chapters Four and Five. Specific discussions regarding the data from each study have already been outlined in their respective chapters. Therefore, this general discussion will attempt to take what was found in each study and discuss it further in the context of how E2 affects DA release, as well as, how E2 facilitates the effects of HAL on D2Rs in the DS. Here, one conceptualization of how estrogens may be contributing to the effects of antipsychotics in the treatment of women with schizophrenia is proposed.

1. Current knowledge of schizophrenia and its treatment

A conceptualization of a striatal DA synapse from what we know so far is illustrated in Figure 1. The synapse is comprised of a presynaptic membrane containing D2 autoreceptors and DA transporters (DATs). It also has a postsynaptic membrane containing DA D1-like and DA D2-like receptors, membrane-bound estrogen receptors (ERα and ERβ), GPER-1, and adenylyl cyclase. The synaptic cleft contains freely moving molecules, in this example, DA. The presynaptic terminal releases DA through the vesicles. The DA molecules in the cleft bind to the receptors at the pre and postsynaptic membrane initiating various signals through the activation of the receptors. For example, activation of D1R triggers binding of G-proteins that then bind to adenylyl cyclase, resulting in production of cAMP, while activation of $D2^{High}$ results in inhibition of cAMP production. Activation of $D2^{High}$ can also commence other second messenger cascades including β-arrestin activation and formulation of a complex between β-arrestin, PP2A, and Akt. The DA molecules in the cleft also bind to the D2 autoreceptors on the presynaptic membrane which trigger inhibition of further synthesis and release of DA by the neuron. The remainder of the DA molecules are quickly taken up into the presynaptic terminal via the DATs. The current treatment for schizophrenia involves targeting the D2Rs located on the postsynaptic membrane.

Figure 2 depicts a model of some of the changes thought to occur in schizophrenia illustrated through a striatal DA synapse. Unlike the typical striatal DA synapse (*see* Figure 1),

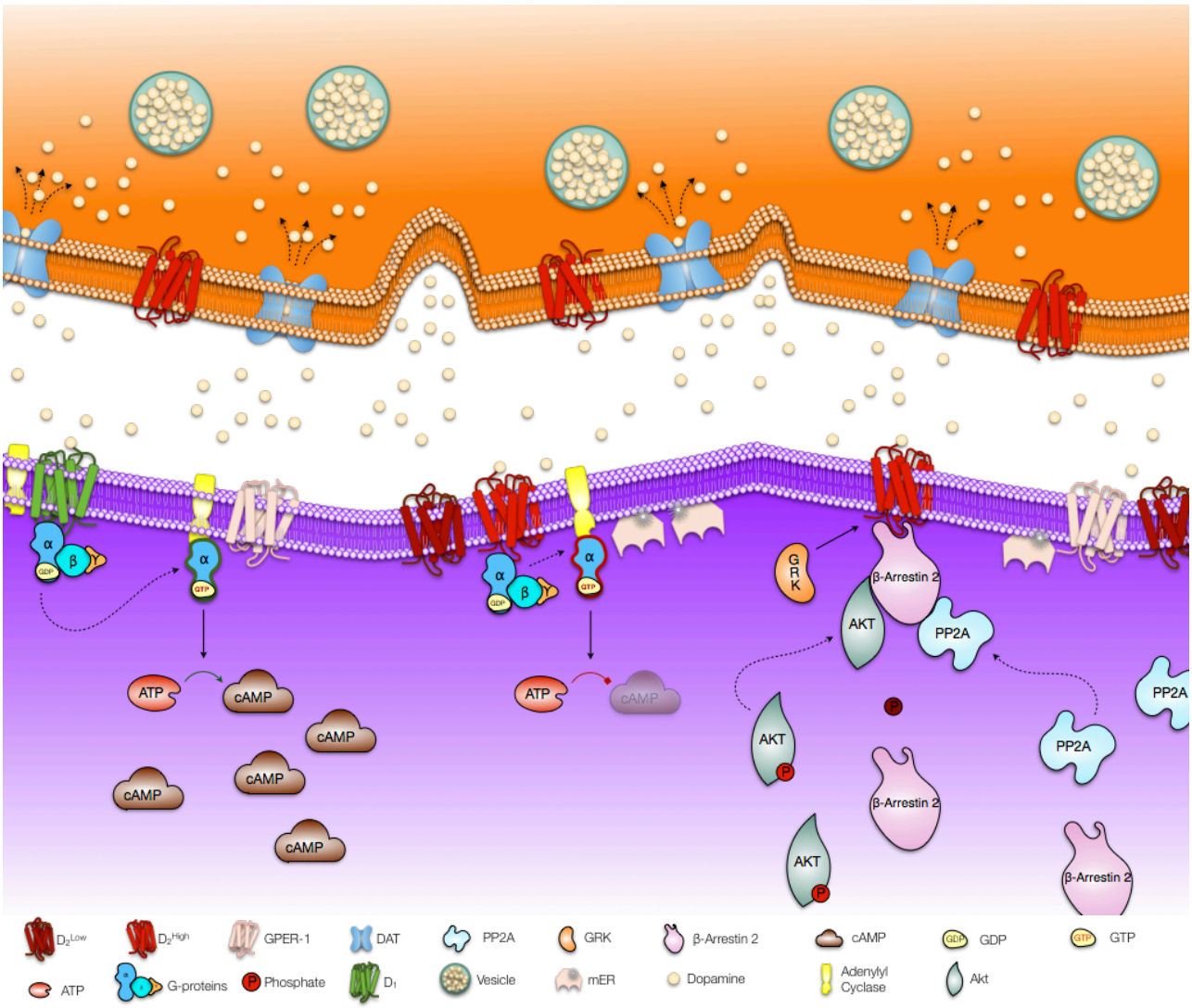

Figure 1. Conceptualization of a striatal DA synapse from what is known up until now. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; PP2A = protein phosphatase 2A; GRK = G-protein receptor kinase; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; ATP = adenosine triphosphate; mER = membrane-associated estrogen receptor; GTP = guanosine triphosphate.

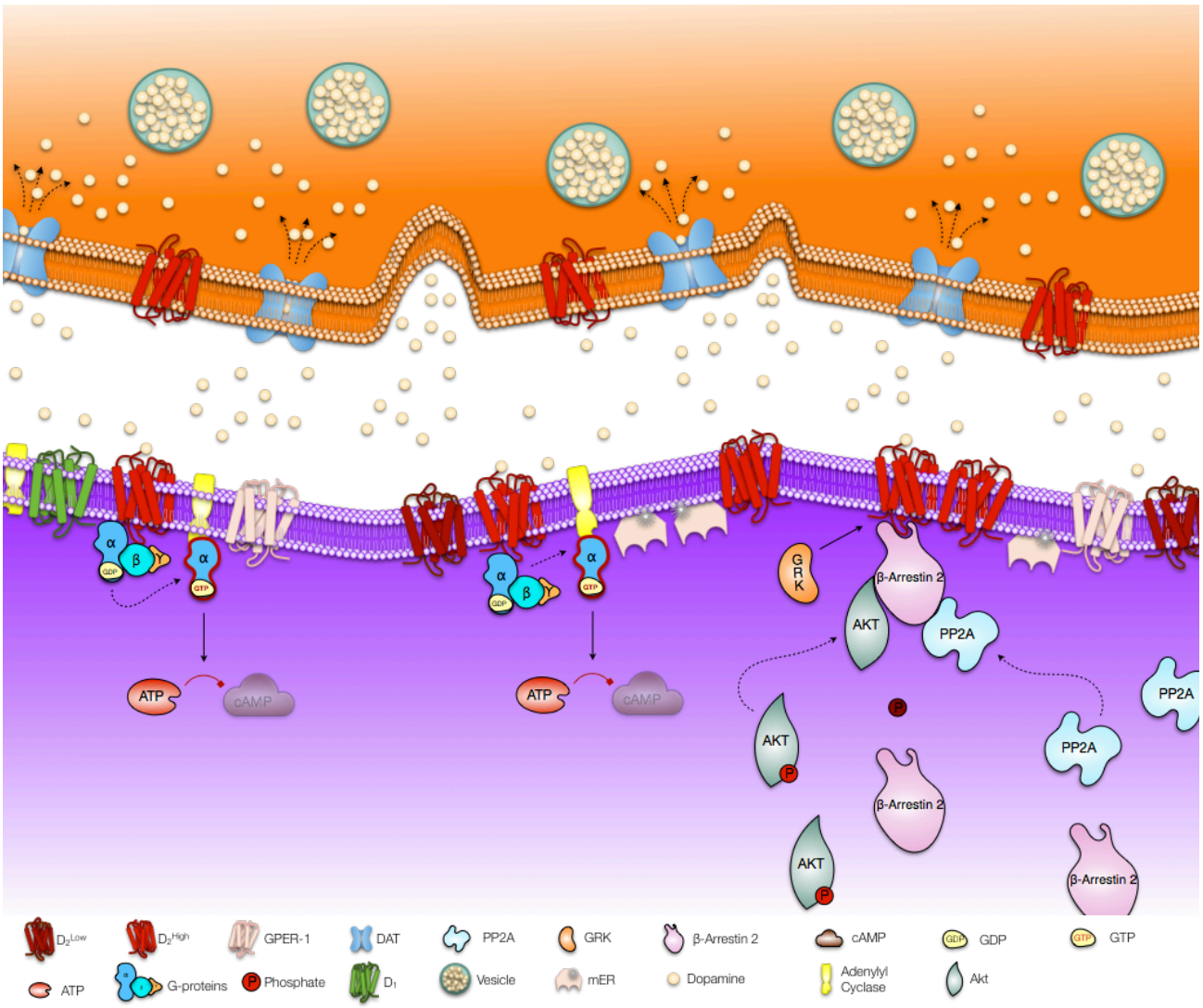

Figure 2. Conceptualization of a striatal DA synapse depicting a model of some of the changes thought to occur in schizophrenia. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; PP2A = protein phosphatase 2A; GRK = G-protein receptor kinase; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; ATP = adenosine triphosphate; mER = membrane-associated estrogen receptor; GTP = guanosine triphosphate.

this synapse's postsynaptic membrane has a higher proportion of D2 receptors present at the postsynaptic membrane (Seeman et al., 2005, 2006). Specifically, there is a greater density of $D2^{High}$ present, resulting in a higher activation of these receptors that further inhibit production of cAMP and formulate a complex between β-arrestin, PP2A, and Akt (Beaulieu et al., 2007). These figures provide a general understanding of what is going on in a typical striatal DA synapse, in comparison to a synapse with some of the changes thought to occur in schizophrenia.

2. Our knowledge of schizophrenia and its treatment in women

Estrogens have been shown to exert protective effects in schizophrenia. Given the relationship between E2 and DA release observed in *in situ* slice preparation studies, *in vitro* studies, and systemic E2 injection studies (Becker and Cha, 1989; Becker, 1990a, 1990b; Castner et al., 1993; Bazzett and Becker, 1994; Xiao and Becker, 1994; Becker and Rudick, 1999), it was only appropriate to further investigate this relationship. Specifically, Chapters Two and Three looked at the effects of E2 on DA transmission. Local infusion of E2 into the DS of anesthetized female rats enhances AMPH-induced tonic striatal DA release as established in Chapter Two. Local infusions of E2 into the mPFC or the SN did not enhance AMPH-induced tonic striatal DA release. To further investigate the effects of E2 on DA transmission, in Chapter Three, it was demonstrated that direct infusion of E2 into the DS increases electrically-evoked phasic striatal DA transients in anesthetized female rats. Although the primary goal of Chapters Two and Three has been well examined, the experiments conducted up until these studies did not investigate the effects of E2 on DA release *in vivo* or identify whether the DA release is tonic or phasic, or both. Findings from Chapters Two and Three established that E2 does in fact increase both tonic and phasic striatal DA release.

The implications of these findings are two-fold. First, the data suggest that E2's effects on DA release are non-genomic. It has been previously debated whether estrogens actions on DA transmission are via the classic nuclear ERs (genomic) or more rapidly through membrane bound receptors (non-genomic), or both. A single physiological dose of E2 (Becker 1990a), or E2 administered to striatal tissue (Becker, 1990b), or subcutaneous administration of E2 or estradiol benzoate (Castner et al., 1993), or both a single or repeated treatment of E2 (Becker and Rudick, 1999) to OVX rats rapidly (within 20 – 30 min) increases AMPH-induced DA release. Given that E2 affects DA release so rapidly, shows that this is a non-genomic effect.

Second, in order for the effect of E2 to be non-genomic, it must be acting at least through its membrane bound receptors. ERα, ERβ, and GPER-1 are localized at the membrane of cells within the DS (Almey et al., 2012). Additionally, these receptors have been observed in the axons and terminals of GABAergic (Almey et al., 2016) and cholinergic neurons, and in glia (Almey et al., 2012). It is likely that E2 is acting on its membrane bound receptors to cause rapid DA release. Our findings of Chapters Two and Three support the theory of non-genomic effects via E2's effects on its membrane bound receptors, given the fact that administration of E2 rapidly increases tonic (Chapter Two) and phasic (Chapter Three) DA release. These results help us understand the effects that E2 has on DA and the mechanism through which this occurs. They also help us clarify the role that E2 may be playing in protecting against schizophrenia and facilitating the effects of antipsychotics.

Figure 3 is an illustration displaying the results of Chapters Two and Three in a typical striatal DA synapse. When the DS receives a local infusion of E2, there is an increase in striatal DA release. Additionally, in Chapter Two, since the DA release was AMPH-induced, DATs on the presynaptic membrane are reversed by AMPH and instead of taking in DA, they release DA out into the cleft. Similarly, in Chapter Three, although DAT is not reversed, the electrical-stimulation of the SN in conjunction with E2 infusion into the DS results in excess DA in the cleft of the striatal DA synapse. This figure provides one conceptualization of the effects of E2 on a typical striatal DA synapse given the findings of Chapters Two and Three.

3. *The Estrogen Paradox*

The original DA hypothesis of schizophrenia posits that this disorder is caused by hyperactive striatal DA transmission. This DA hypothesis of schizophrenia and the results from Chapters Two and Three combined produce paradoxical findings. If E2 in fact does help protect against schizophrenia, how can E2 also be increasing DA release when the original hypothesis states that this disorder is caused by hyperactive striatal DA transmission? A recent study in our laboratory found that high E2 in conjunction with HAL reduces AMPH-induced DA release in AMPH-sensitized female rats (Madularu et al., 2014). Given that HAL, a typical antipsychotic, is a D2R antagonist, and E2 alters D2R affinity state, it is possible that E2's protective effects against schizophrenia may be through its effects on D2R, in conjunction with HAL. As such,

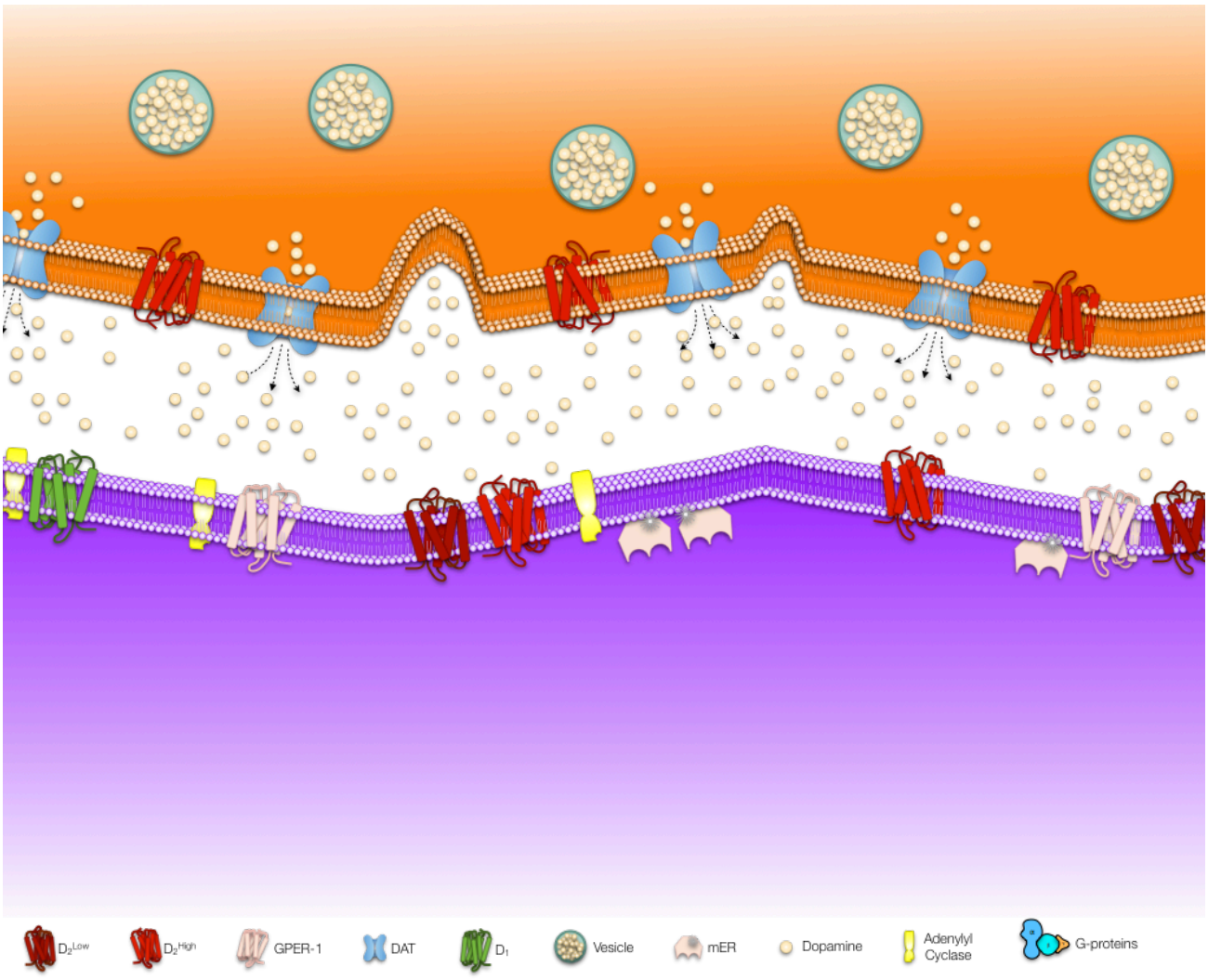

Figure 3. An illustration displaying the results of Chapters Two and Three in a typical striatal DA synapse. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; mER = membrane-associated estrogen receptor.

Chapters Four and Five investigated the mechanism through which E2 affects DA transmission by examining its relationship with the D2R.

4. Estrogens in conjunction with antipsychotics are a better treatment: Why?

It has been shown that unmedicated individuals with schizophrenia have a greater density of $D2^{High}$ present in the brain than those without schizophrenia or other forms of psychoses (Seeman, 1987). Animal models mimicking symptoms of schizophrenia also have shown an increase of 360% in the density of $D2^{High}$ (*for review, see* Seeman et al., 2005). Interestingly, as previously stated, estrogens can alter the affinity state of the D2R. For example, the affinity state of D2R fluctuates across the estrous cycle of a rat with the most $D2^{High}$ during diestrus 2, when E2 levels are low, and the most $D2^{Low}$ during estrus and proestrus, when E2 levels are high (Di Paolo et al., 1988). Given these findings, Chapter Four investigated the effects of E2 on D2R and its affinity state in order to identify a potential mechanism through which estrogens in conjunction with antipsychotics are a better treatment for schizophrenia. This study examined the effects of E2 replacement in AMPH-sensitized female rats treated with HAL or saline on D2R affinity state. It was established that HAL and E2 increases the affinity of $D2^{High}$ and $D2^{Low}$ receptors, and most importantly, *decreases* the proportion of D2Rs in the high affinity state. These findings implicate that E2 is facilitating the effects of HAL on D2R. As mentioned earlier, individuals with schizophrenia have a greater density of $D2^{High}$. This is one mechanism through which E2 in conjunction with antipsychotics is a better treatment for schizophrenia than antipsychotics alone since E2 has suppressing effects on the D2R affinity state.

Figures 4 and 5 illustrate one conceptualization of this mechanism when taking into account the findings of Chapter Four. Figure 4 depicts a typical striatal DA synapse that has undergone HAL treatment. HAL, a D2R antagonist, blocks a number of the D2Rs including the D2 autoreceptors on the presynaptic membrane and the D2Rs on the postsynaptic membrane, preventing activation of these receptors. An important note here is that, in comparison to the synapse depicted in Figure 2, this synapse contains even a greater number of $D2^{High}$ due to DA supersensitivity (*for review*, *see* Seeman, 2006, 2010, 2011). As such, the unblocked D2Rs are readily bound by DA resulting in activation of G-proteins and second messenger cascades. Figure 5 conceptualizes what may be happening at the striatal DA synapse when both HAL and E2 are administered. The combination of the two decrease the proportion of $D2^{High}$. HAL blocks

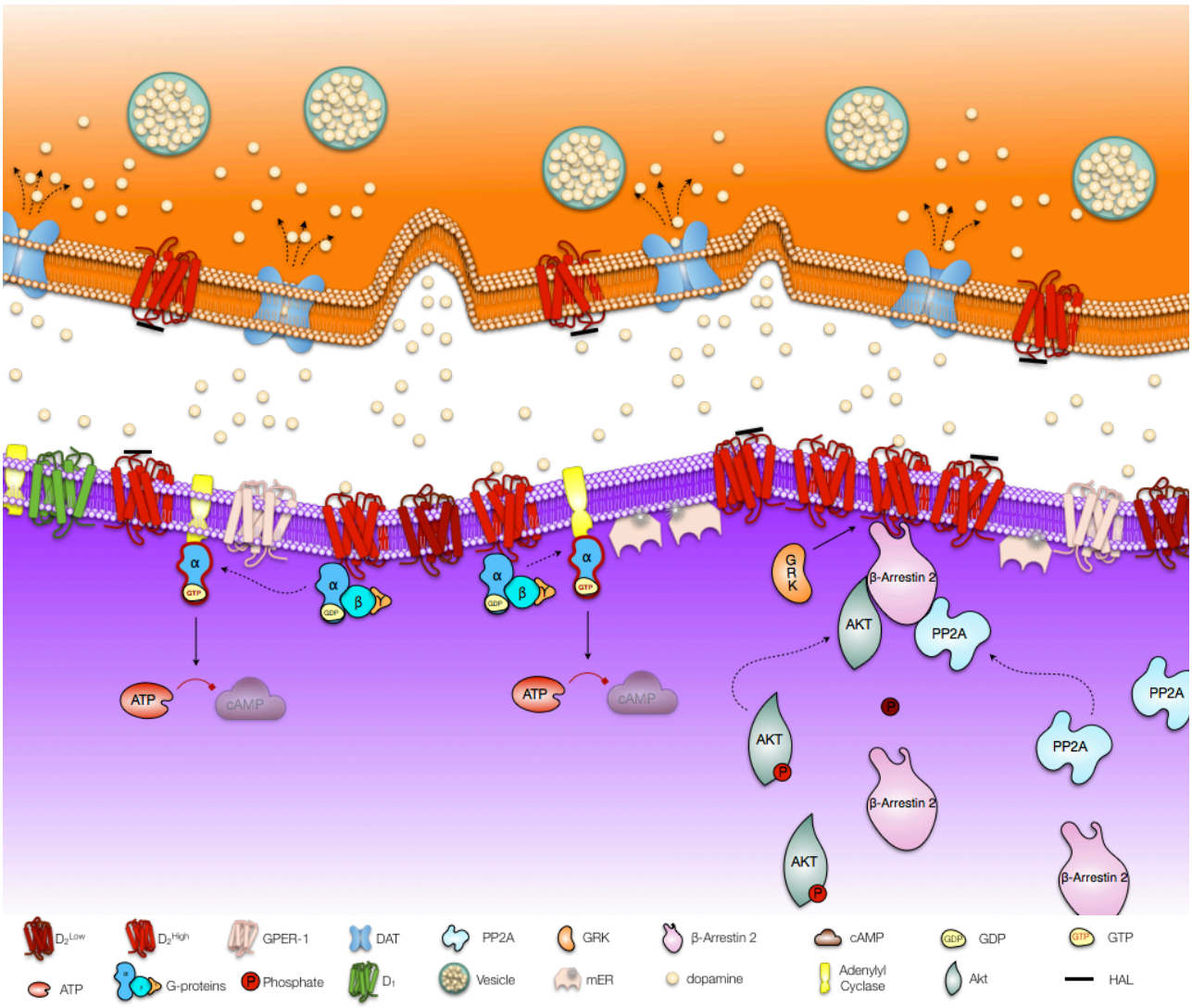

Figure 4. Conceptualization of a striatal DA synapse that has undergone HAL treatment. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; PP2A = protein phosphatase 2A; GRK = G-protein receptor kinase; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; ATP = adenosine triphosphate; mER = membrane-associated estrogen receptor; GTP = guanosine triphosphate; HAL = haloperidol.

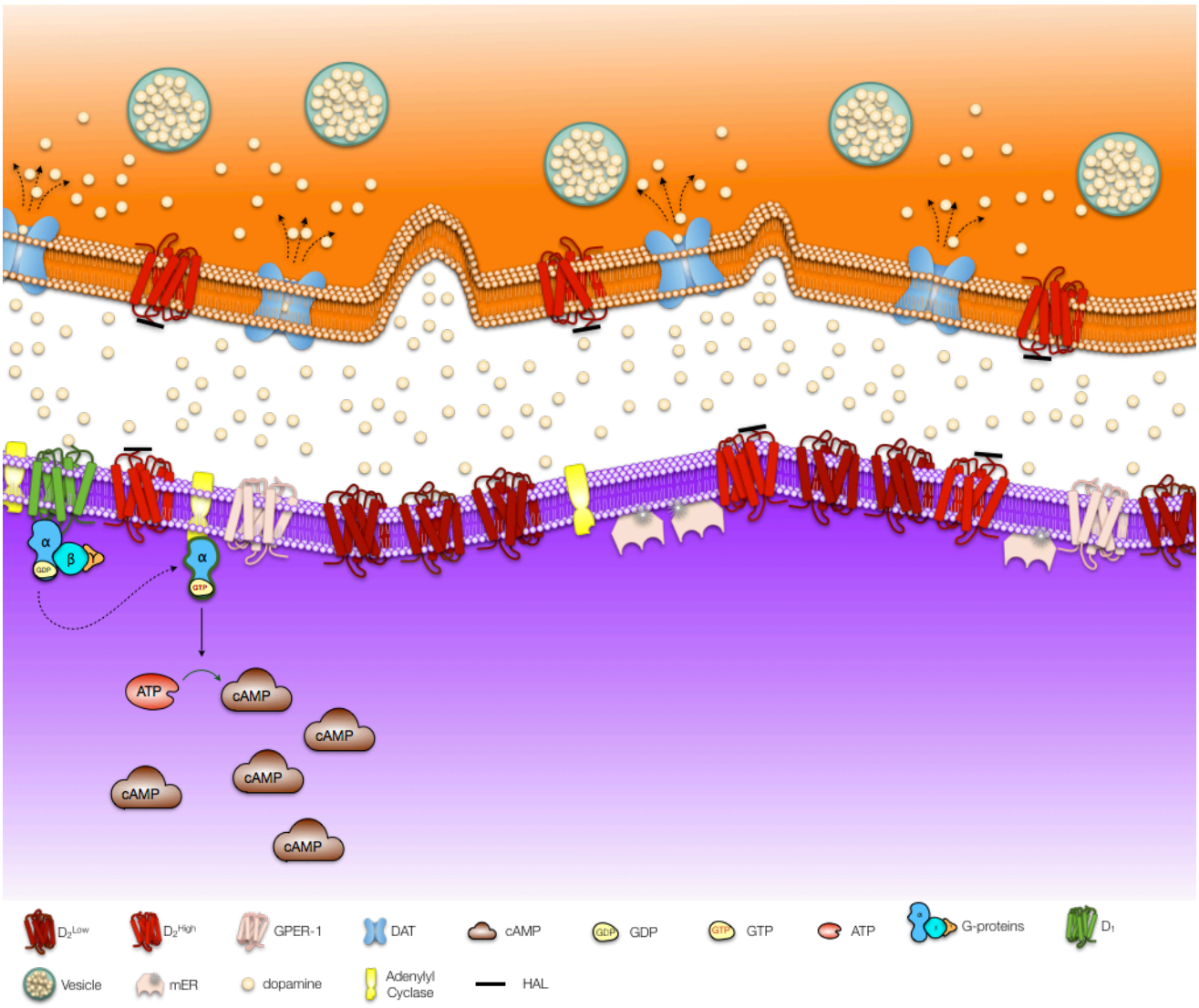

Figure 5. Conceptualization of what may be happening at the striatal DA synapse when both HAL and E2 are administered. GPER-1 = G-protein-coupled estrogen receptor-1; DAT = dopamine transporter; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; ATP = adenosine triphosphate; mER = membrane-associated estrogen receptor; GTP = guanosine triphosphate; HAL = haloperidol.

D2Rs while E2 converts the unblocked $D2^{High}$ into $D2^{Low}$, as shown by Levesque and Di Paolo (1988). Although E2 also increases extracellular DA as found in Chapters Two and Three, E2 and HAL's effects decrease activation of the D2R. It is this specific effect of E2 on the D2R that underlies a possible mechanism through which E2 in conjunction with antipsychotics may be a better treatment for schizophrenia.

Blocking of the D2R prevents inhibition of cAMP, and possibly formation of the β-arrestin, PP2A, and Akt complex. In order for the formation of this complex to occur, pAkt has to be dephosphorylated, potentially making it inactive. Reduced Akt activity caused by excessive activation of D2Rs may be implicated in schizophrenia. As such, Chapter Five examined the effects of E2 and HAL on Akt, pAkt, and β-arrestin levels in AMPH-sensitized female rats. The purpose of this experiment was to further investigate the mechanism through which E2 affects DA transmission by targeting one of the second messenger cascades and the proteins involved in it. No significant differences were observed in Chapter Five. The null results in Chapter Five suggest that the mechanism through which E2 in conjunction with antipsychotics is a better treatment for schizophrenia does not include this specific second messenger cascade. It is possible that the effects of E2 on the D2R affinity state do not alter this second messenger cascade, and in turn impact the density of Akt, pAkt, or β-arrestin.

5. Caveats

Although the experiments in this thesis focused specifically on the DS as the main brain region of interest when investigating the effects of E2 on DA transmission, this has not been the case in some of the initial studies in the past. Previous studies did not differentiate between dorsal and VS (also referred to as the NA). For example, fMRI and PET studies were not able to distinguish between the dorsal and VS. Thus, it was common practice to measure dorsal and VS as one region, the striata. However, it is well known that the dorsal and VS are two different brain regions. In the context of schizophrenia alone, positive symptoms are thought to be a result of DA hyperactivity in the VS (*for review, see* Neve, 2010). Whereas the extrapyramidal side effects produced by the typical antipsychotics are a result of blocking DA transmission in the DS (*for review, see* Neve, 2010). To remain consistent with the seminal work done by Seeman and colleagues, all of the experiments in this thesis targeted specifically the DS. This brain region has been well studied in the context of schizophrenia and its treatment, targeting the D2R. It is

important to note that although the current thesis targeted the DS, the VS is also an essential brain region of interest when studying schizophrenia. As such, findings of the studies targeting the DS should keep this caveat in mind.

DA release in Chapters Two and Three was measured in anesthetized rats. In Chapter Two, Isoflurane was used as an anesthetic, whereas in Chapter Three, urethane was used as an anesthetic. The use of anesthesia could have affected the outcome of the results. Although we are not aware of any affects, it is possible that the use of Isoflurane or urethane could alter DA transmission.

Chapters Four and Five used AMPH-sensitization as the animal model mimicking symptoms of schizophrenia in order to study some aspect of the disorder. Although this animal model is commonly used to study some of the symptoms of schizophrenia, it is not the same as studying the disorder itself. There is an obvious discrepancy between studying animal models that mimic schizophrenia and studying the disorder in humans. As such, the findings of studies using animal models in the DS should keep these caveats in mind.

6. Future directions

Future studies should further investigate the relationship between E2 and DA and the mechanisms through which E2 affects DA transmission. Although Chapters Two and Three showed that E2 rapidly increases striatal DA release, additional studies should determine how E2 increases striatal DA release. For example, it is possible that the rapid effects of E2 on striatal DA release could be due to E2 action at the mERs on the terminals of GABA neurons via GABA-mediated disinhibition by E2. Membrane-associated ERs are also found on cholinergic neurons in the DS, which could also be contributing to these effects by E2.

Chapter Four found that E2 and HAL decrease the proportion of $D2^{High}$ in the DS, providing a potential mechanism through which E2 affects DA transmission. However, additional research needs to be conducted to address what is E2 physically doing to convert $D2^{High}$ into $D2^{Low}$. Future studies should investigate whether E2 is acting directly on the D2R to alter its affinity state, or if it is acting on its own membrane-bound receptors to then affect the D2R affinity state. If E2 is acting on its own receptors, which specific receptors is it interacting with to alter D2R affinity state should also be investigated. In such cases, different E2 receptor agonists and antagonists could be used to further study the effects of E2 on DA release, and the

mechanisms behind how this occurs. Additionally, given the effects E2 has on the D2R and its affinity state, what kind of conformational changes are the receptors undergoing, if any.

The experiments in this thesis used one specific dose to investigate the effects of E2 on DA release and the mechanism through which E2 affects DA transmission. Future studies should examine different doses of E2 on DA transmission. The dose of E2 is vital for DA transmission since the change in E2 levels throughout the estrous cycle directly impacts the density and conversion of the D2R and its affinity state. Additionally, the results of Chapters Four and Five are only specific to the effects of E2 in conjunction with typical antipsychotics. Future studies should investigate the effects of E2 in conjunction with atypical antipsychotics on D2R affinity state and its second messenger cascades.

Currently, the primary focus of pharmacological treatment for schizophrenia incorporates the antagonism of D2Rs. In particular, both typical and atypical antipsychotic drugs are D2R antagonists. With all of the focus on D2Rs for the treatment, the critical role of D1Rs in this disorder has been neglected. Due to this, surprisingly, pharmacological treatments for schizophrenia do not encompass D1Rs. D1Rs are important for a variety of central nervous system functions, including motor control, learning and memory (Missale et al., 1998), and memory-related processes (Neve, 2010). Research involving D1Rs supports evidence that dysregulation of the D1Rs in the PFC may be a contributing factor to the cognitive deficits seen in individuals with schizophrenia. For example, positron emission tomography (PET) ligand binding studies targeting the PFC have reported changes in the D1R binding affinity associated with negative symptoms and cognitive performance (*for review, see* Goldman-Rakic et al., 2004). Okubo et al (1997) found that down-regulation of D1R binding in the PFC of untreated individuals with schizophrenia was positively correlated with impairment in performance on a test that measures central executive functioning, the Wisconsin Card Sorting Task (WCST; Kashima, 1991). Specifically, in healthy young adult monkeys, chronic treatment of either typical or atypical antipsychotics administered at therapeutic doses resulted in a down-regulation of D1Rs in the PFC (Lidow and Goldman-Rakic, 1994). Furthermore, Castner et al (2000) found that chronic treatment of typical antipsychotics induced down-regulation of the D1Rs in the PFC producing severe impairments in working memory of young adult monkeys. However, these deficits were reversed with a short-term co-administration of the D1R agonists (Castner et al., 2000). Goldman-Rakic and colleagues found that an optimal level of D1Rs are required in the

PFC for sufficient working memory performance (Neve, 2010). If DAergic transmission is low in the PFC, increasing D1R stimulation helps improve working memory. If the DAergic transmission is high in the PFC, decreasing D1R stimulation helps improve working memory. Therefore, D1R activation levels follow an inverted "U" shape in regards to working memory performance, where too much or too little D1R stimulation would result in an impairment of working memory performance (Goldman-Rakic et al., 2000). These findings provide evidence that the D1R is important in functioning of the PFC and its activity can alter working memory and executive functioning, two vital functions found to be impaired in individuals with schizophrenia experiencing cognitive symptoms. Taken together, evidence suggests that the D1R may play a valuable role and may be an important target for improving cognitive function in schizophrenia. Future studies should investigate the role of the D1R and the effects E2 has on the D1R, in the context of schizophrenia.

Lastly, the focus of this thesis was strictly on E2. As such, additional work is required to examine the contribution of other ovarian hormones in females, such as progesterone, and the primary sex hormone in males, testosterone, in the context of schizophrenia and their role in its treatment.

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