Membrane-associated estrogen receptors and cognition in female rats.

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ABSTRACT

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There are sex differences in dopamine-dependent diseases and behaviours, and accumulating evidence suggests that estrogens are partially responsible. This thesis examines the effects of estrogens on dopamine-dependent cognitive processes. Some of these cognitive processes are affected in schizophrenia, so these experiments also examined the combined effects of estrogen and the antipsychotic drug haloperidol on these cognitive processes.

The first study in this thesis examined the effects of estrogens and haloperidol on selective attention, measured in a latent inhibition paradigm. The result of these experiments demonstrated that estrogens have detrimental effects on latent inhibition, but facilitate an acute dose of haloperidol to restore latent inhibition in female rats. The next two studies extended these findings to two other cognitive processes negatively affected in individuals with schizophrenia: perseveration and reversal learning. Estrogens alone had no effect on perseveration or reversal learning in amphetamine sensitized female rats, but did fascilitate haloperidol to reduce perseveration and improve reversal learning.

Previous research has observed very low levels of estrogen receptors in the striatum, nucleus accumbens and prefrontal cortex, regions that mediate the majority of dopamine-dependent cognitive processes. Immunoelectron microscopy was used to examine estrogen receptors in these regions to provide a mechanism for estrogens' effects on dopamine dependent behaviour. Immunohistochemistry was used to examine the distribution of estrogen receptors, ERα, ERβ, and GPER1, demonstrating that these receptors are observed primarily at presynaptic extranuclear sites and in glia in the striatum, nucleus accumbens and prefrontal cortex. In the striatum a small proportion of ERα and GPER1 are localized to cholinergic interneurons and a larger proportion of these receptors are observed in GABAergic neurons in the striatum. In the accumbens a low proportion of ERα and GPER1 were localized to catecholaminergic neurons, and a greater proportion of these receptors were observed in GABAergic neurons. The final experiment in this thesis examined whether binding at membrane-associated estrogen receptors

could rapidly affect dopamine-dependent cognition. Increasing estrogens in the prefrontal cortex rapidly biases female rats towards use of place memory, indicating that binding at membrane-associated estrogen receptors can rapidly affect dopamine-dependent cognitive processes.

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CONTRIBUTION OF AUTHORS

Chapter 2: These experiments were conceived by Anne Almey in conjunction with Wayne Brake based on previous research in this lab by Mathew Quinlan and Wayne Brake. Anne Almey conducted these experiments under the supervision of Wayne Brake with some assistance from Nada Hafez and Arne Hantson.

Chapter 3: These experiments were conceived by Anne Almey in conjunction with Wayne Brake. Anne Almey conducted these experiments under the supervision of Wayne Brake in collaboration with undergraduate thesis students: Nada Hafez, Cynthia Mancinelli, Lauren Arena, Joshua Oliel, Lukas Henning, and Aleks Tsanev.

Chapter 4 A and B: These experiments were conceived by Anne Almey and Wayne Brake with methodological expertise provided by Teresa Milner. Anne Almey conducted these experiments under the co-supervision of Teresa Milner and Wayne Brake, while Edward Filardo provided the GPER1 antibody used in these experiments.

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Chapter 6: This study was conceived by Anne Almey in conjunction with Wayne Brake. Anne Almey conducted the behavioural experiment under the supervision of Wayne Brake in collaboration with undergraduate thesis students Kyla Bertram and Elizabeth Cannell. Anne Almey conducted the electron microscopy experiments under the co-supervision of Teresa Milner and Wayne Brake, while Edward Filardo provided the GPER1 antibody used in these experiments.

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LIST OF ABBREVIATIONS

17β estradiol……E2
Acetylcholine……..…ACh
Cyclic AMP Response Element Binding Protein……..CREB
Dorsal Striatum…….....STR
Estrogen receptor…….....ER
Estrogen receptor alpha…….ERα
Estrogen receptor beta……...ERβ
Estrogen Response Element……..…ERE
G protein-coupled estrogen receptor 1…….GPER1
Gamma-Aminobutyric acid..…….GABA
Haloperidol……...HAL
Immunoreactive……..…..IR
Latent Inhibition…….....LI
Medial Prefrontal Cortex……....mPFC
Membrane-associated estrogen receptor……..... mER
Not Preexposed…….....NPE
Nucleus Accumbens…….NAc
Ovariectomized……...OVX
Preexposed……..…PE
Prefrontal Cortex……..….PFC
Saline……....SAL
Tyrosine Hydroxylase……....TH
Vesicular Acetylcholine transporter……...VAChT

CHAPTER 1:
GENERAL INTRODUCTION

ESTROGEN RECEPTORS IN THE CENTRAL NERVOUS SYSTEM AND THEIR IMPLICATION FOR DOPAMINE-DEPENDENT COGNITION IN FEMALES

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The effects of estrogens on cognition, and the mechanisms through which these effects are achieved in the brain, are more varied and complex than was initially believed. Estrogens are a class of steroid hormones that include estrone, estriol, and estradiol (E2), the last of which is the most potent estrogen in female mammals during their reproductive years. There is substantial research examining the role of estrogens in cognition (see Luine, 2014 for review). Interestingly, although estrogens have also been implicated in dopamine-dependent cognition, the brain regions important for this (viz. the dorsal striatum (STR), nucleus accumbens (NAc), prefrontal cortex (PFC) and hippocampus) have relatively sparse nuclear labelling for estrogen receptors (ERs; Milner et al 2001, Mitra et al 2003, Shughrue et al 1998, Weiland et al 1997). Some effects of estrogens on dopamine transmission in these regions occur over a relatively long time course (> 10h) and are mediated by nuclear ERs (Luine et al 1998, Korol and Kolo 2002, Quinlan et al 2010) but other estrogen effects are too rapid to occur through binding at the nuclear ERs (Almey et al 2014, Becker and Rudick 1999, Thompson and Moss 1994). This review examines the evidence for membrane-associated estrogen receptors (mERs), the role of both nuclear ERs and mERs in dopamine-dependent cognition, and recent immunoelectron microscopy research localizing mERs to the STR, NAc, PFC, and hippocampus.

Following the discovery of estrogens in 1929 (Butenandt 1929), research on this class of steroid hormones focused on their role in reproduction and the menstrual/estrous cycle in females (Doisy 1972). In 1966, an ER was characterized in breast and uterine tissue (Toft and Gorski 1966), and this ER was also localized to brain regions typically associated with endocrine or reproductive functions, such as the hypothalamus (for review see McEwen and Alves 1999). This receptor, now known as estrogen receptor alpha (ERα), was observed primarily in cell nuclei, typical for steroid hormone receptors. In the mid-1990s a second ER, estrogen receptor beta (ERß), was discovered, which also was localized to cell nuclei (Kuiper et al 1996). Both ERα and ERß are expressed in the uterus, breast tissue, testicles, prostate, cardiovascular system, and to a lesser extent in the bone and the lungs (Rollerova and Urbancikova 2000). More relevant to this review, these ERs are expressed in the pituitary and many brain regions, including the hypothalamus, the hippocampus, the amygdala, and the PFC, among others (Kuiper et al 1998, Montague et al 2008, Shughrue et al 1998, Shughrue and Merchenthaler 2001, Spencer et al 2008). Estrogen activation of ERs either directly or indirectly contribute to certain diseases and disorders (Brann et al., 2007) and have numerous behavioural effects including:

increasing agonistic behaviours, improving performance in spatial learning and memory tasks, and initiating copulatory behaviours (Luine et al 1998, Clipperton-Allen et al 2011, Clipperton Allen et al 2010, Gervais et al 2013, Almey et al 2014, Brann et al 2007, Cornil and Charlier 2010).

The original conceptualization of ERα and ERβ was as typical steroid/nuclear receptor located in the cytoplasm of cells when not activated. When estrogens bind to these receptors, the newly formed receptor-ligand complexes dimerize and translocate to the nucleus where they bind to estrogen response elements (EREs) on DNA (Kumar and Chambon 1988) to regulate the transcription of proteins (Nilsson et al 2001). It is difficult to predict the effects of binding at an ERE, since EREs can have different transcriptional effects, and numerous co-activators and co-repressors alter the transcriptional effects of binding at EREs (Kuiper et al 1996, Rollerova and Urbancikova 2000). Nonetheless, there is evidence that estrogens alter the production of multiple proteins in the central nervous system, including growth factors (Varea et al 2010, Woolley 1999), cytokines (Kovacs et al 2002), and apoptotic factors in the brain (Kiess and Gallaher 1998, Vasconsuelo et al 2011). In addition to acting through these nuclear ERs to elicit long-term effects, ERs can be found at the cell membrane, where estrogen-binding induces rapid effects such as altering membrane permeability (Fu and Simoncini 2008, Wong and Moss 1992) and activating second messenger cascades (Edwards and Boonyaratanakornkit 2003, Fu and Simoncini 2008).

The earliest evidence for membrane ERs (mERs) was from Pietras and Szego (1975), who demonstrated that application of E2 to endometrial cells causes rapid depolarization as a result of an increase in intracellular Ca^{2+} influx. The authors then used subcellular fractionation techniques to isolate the cell membrane from the cytoplasm, and demonstrated that ERα is present in membrane fractions from endothelial tissue suggesting that ERα is associated with cell membranes (Pietras and Szego, 1980). Shortly afterwards, it was shown that the application of E2 to parvocellular neurons in the medial preoptic area, arcuate nucleus, and the ventromedial hypothalamus resulted in fast hyperpolarization of these cells (Kelly et al 1980, Kelly et al 1976). These findings inspired further research on these rapid effects of estrogens, which revealed that application of estrogens to neurons from the arcuate and ventromedial hypothalamus or the amygdala resulted in hyperpolarization of these cells in the presence of transcription blockers (Kelly et al 1980, Minami et al 1990, Nabekura et al 1986). Additionally,

the membrane-impermeable E2-bovine serum albumin (BSA) conjugate binds to receptors in the hypothalamus, cerebellum, and olfactory bulb (Zheng and Ramirez 1997). Since estrogens' long-term effects cannot occur rapidly, in the presence of transcription blockers, or via E2-BSA, these early studies concluded that estrogens must bind at some then-unknown membrane-associated ER to elicit these rapid effects.

1. Membrane associated estrogen receptors

1.1 ERα and ERβ: steroid receptors in a novel location

There is now evidence that ERα and ERß are both found at the cell membrane, as well as in nuclei and in the cytoplasm, where they were originally localized. These two receptors are found as either homo- (ERα-ERα or ERβ-ERβ) or hetero- (ERα-ERβ) dimers at the membrane, and they are membrane-associated, but not actually embedded within the cell membrane (Boonyaratanakornkit and Edwards 2007). mERα and mERβ can induce a number of intracellular events typically induced by activating G protein-coupled receptors. mERs are thought to activate G protein-coupled receptors to regulate L-type Ca^{2+} channels and activate protein kinase A (PKA), protein kinase C (PKC), and mitogen activated protein kinase (MAPK) signalling cascades (Coleman and Smith 2001, Fu and Simoncini 2008, Yang et al 2008). The mechanisms through which ERα and ERβ become associated with the cell membrane remain unclear, but two are believed to be pivotal: the post translational lipid modification of these ERs, and their interaction with membrane/cytoplasmic scaffolding proteins (Boonyaratanakornkit 2011). Note that research on ER membrane association has predominantly focused on ERα.

The primary form of lipid modification associated with mERs is palmitoylation. Palmitoylation refers to the addition of palmitic acid to specific residues of proteins, typically membrane-associated proteins (Basu 2004). If palmitoylation is inhibited in hippocampal cell cultures, rapid estrogen-induced phosphorylation of cyclic AMP response element binding protein (CREB) is eliminated (Meitzen et al 2013). Furthermore, palmitoylation occurs at specific cysteine sites of ERα and ERβ receptors; when these palmitoylation sites are mutated, rapid estrogen-induced CREB phosphorylation and activation of MAPK and PI3 kinase are blocked (Meitzen et al 2013, Pedram et al 2002). Additionally, E2-induced decreases in synaptosomal membrane-associated ERα in the hippocampus occur through depalmitoylation (Tabatadze et al 2013). Together, these findings indicate that palmitoylation of specific cysteine

residues of ERs is critical for the rapid effects of membrane-associated ERs. A truncated version of ERα was discovered in endothelial cells; this truncated ERα is 46-kDa as opposed to the typical 67-kDa ERα. This 46-kDa version of ERα is preferentially palmitoylated, and is more effective at rapidly activating endothelial nitric oxide synthase than the traditional ERα (Li et al 2003), but does not mediate transcriptional responses (Figtree et al 2003). This suggests that there is a smaller isoform of ERα primarily associated with the cell membrane.

Interactions between ERs and certain scaffolding proteins also are believed to play a critical role in the association of ERα and ERβ with the cell membrane. The scaffolding proteins receiving the most attention for their role in facilitating membrane-association of ERs are caveolins (Boonyaratanakornkit 2011). Caveolins are the primary structural components of caveolae, which are 50-100nm invaginations of the cell membrane. The structure of caveolae is thought to promote protein-protein interactions and integrate receptors and signaling molecules to facilitate rapid and specific signal transduction (Okamoto et al 1998). ERα is localized in caveolar subfractions of endothelial plasma membranes (Chambliss et al 2000), and confocal microscopy has observed extensive colocalization of caveolins and ERα (Pedram et al 2002). Caveolins are hypothesized to facilitate transport of ERα from the cytoplasm to caveolae, as endothelial cells expressing caveolin have a significantly higher ratio of membrane to cytoplasmic ERα (Pedram et al 2002), and knocking down caveolin in the arcuate nucleus of the hypothalamus reduces the expression of mERα (Christensen and Micevych 2012). Additionally, estrogens affect the production of caveolins; application of physiological levels of E2 significantly increases levels of caveolin and increases caveolin-ERα associations (Razandi et al 2002). These results suggest that estrogens increase levels of caveolin, which in turn facilitates transport of ERα to the cell membrane (Razandi et al 2002). Interestingly, when the palmitoylation site on ERα is mutated, the physical association between ERα and caveolin is reduced (Pedram et al 2007); this suggests that palmitoylation of ERα facilitates interactions between this receptor and caveolins.

Palmitoylation of mERs and associations with caveolins allow these receptors to associate with cell membranes, but does not explain the effects of binding at these mERs on neuronal transmission. The rapid effects of estrogens have been shown to be sensitive to G protein manipulation, which suggests that mERα and mERβ may be able to alter G protein receptor activity (Kelly and Wagner 1999). Research suggests that binding at mERα and mERβ

stimulates metabotropic glutamate receptors (mGluRs; Meitzen and Mermelstein 2011). mGluRs are G-protein-coupled receptors categorized into three families: mGluRI (mGluR1 and 5) that are Gq receptors, and mGluRII (mGluR2 and 3) and III (mGluR4, 6, and 7), which are Gi/o receptors (Niswender and Conn 2010). In cultured hippocampal and striatal cells, application of E2 increases CREB phosphorylation within 30 seconds, an effect that is mediated by ERα (Boulware et al 2005, Grove-Strawser et al 2010). In cultured hippocampal neurons this effect of E2 is replicated by applying an mGluR1 agonist, and blocked by applying an mGluR1 antagonist to the cells (Boulware et al 2005). In contrast, in striatal neurons the E2-induced increase in CREB phosphorylation is mimicked by mGluR5 agonists and blocked by mGluR5 antagonists (Grove-Strawser et al 2010). Additionally E2 can have bidirectional effects on CREB phosphorylation in these brain regions. In hippocampal cultures E2 also inhibits CREB phosphorylation via binding at ERα and ERβ, an effect blocked by mGluR2 antagonists and mimicked by mGluR2 agonists (Boulware et al 2005). In striatal cultures the E2-induced decrease in CREB function is mediated by mGluR3 receptors (Grove-Strawser et al 2010). More recently it was shown that binding at ERα in the CA1 of the hippocampus activates mGluR1, mobilizing components of the endocannabinoid system, leading to reduced gamma-Aminobutyric-acid (GABA) release (Tabatadze et al 2013). The authors interpreted these findings to suggest that some of the rapid effects of E2 in the hippocampus and STR are mediated by mER interactions with different members of the mGluRI and mGluRII receptor families (Meitzen and Mermelstein 2011).

mGluRs are frequently associated with caveolins, and inhibiting caveolin expression or activity inhibits E2 effects on CREB phosphorylation, indicating that the association between E2 and mGluRI and/or mGluRII is dependent on caveolins (Boulware et al 2007). Taken together, these studies suggest that the classical ERs are palmitoylated, which may promote the interaction between these ERs and caveolins (see Fig 1; Meitzen et al 2013). Caveolins facilitate transport of mERs to caveolae where these receptors form associations with mGluRs; binding at these mERs alters CREB activity via activation of mGluRs (Meitzen et al 2013). There are other mechanisms involved in ER-membrane associations; multiple proteins including the adapter protein Shc, the calmodulin binding protein striatin, and the modulator of non-genomic activity of ERs, have also been implicated in the membrane association of ERs (Boonyaratanakornkit 2011, Boonyaratanakornkit and Edwards 2007). However, the palmitoylation of mER, and mER

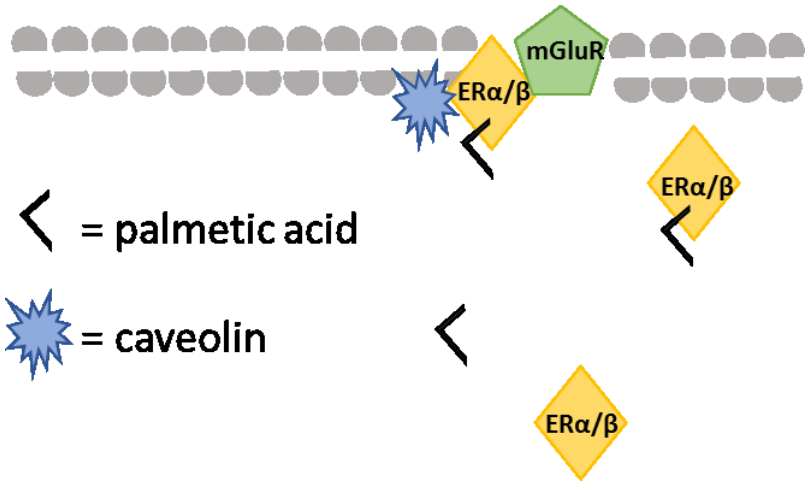

Figure 1. Schematic representation of how ERα or ERβ may be bound to the neuronal membrane via associations with caveolin following palmitoylation of the receptor.

associations with caveolins and mGluRs, provide the most complete explanation for how mERs become associated with the cell membrane, and occur in dopamine-innervated regions (Boulware et al 2005, Grove-Strawser et al 2010, Meitzen and Mermelstein 2011, Huang and Woolley 2012).

1.2 GPER1: a membrane-bound G protein-coupled estrogen receptor

In addition to mERα and mERβ, a third membrane-associated ER was discovered. An orphan G protein-coupled receptor, G protein-coupled receptor 30 (GPR30; a protein migrating at 30 kDa) is now designated as G protein-coupled estrogen receptor 1 (GPER1). GPER1 first was identified in breast tissue (Carmeci et al 1997), and has a single binding site specific to estrogens (Prossnitz et al 2008). Initially, GPER1 was observed at the endoplasmic reticulum in neurons, so it was hypothesized that binding at GPER1 modulated the effects of estrogens at ERα or ERß (Revankar et al 2005, Sakamoto et al 2007). GPER1 is also found on the plasma membrane of cells in the hippocampus and hypothalamus, indicating that binding at GPER1 could have direct effects on neuronal transmission in these brain regions (Funakoshi et al 2006, Prossnitz et al 2008, Waters 2015). The effects of binding at GPER1 are not fully elucidated, but evidence demonstrates that application of E2 to COS (fibroblast-like cell line) or HeLa cells transfected with GPER1 rapidly increases Ca^{2+} influx (Bologa et al 2006, Funakoshi et al, 2006). Additionally, binding at GPER1 activates the phosphoinositide 3-kinase second messenger-signalling cascade (Prossnitz et al 2008), the MAPK signalling cascades (Filardo et al 2000) and the PKA signalling cascade (Fu and Simoncini 2008). Recently, we found that GPER1 in the hippocampus interacts with the PSD-95 and the spine scaffolding protein SAP97 that would position GPER1 for rapid signalling at the spine synapse (Akama et al 2013, Waters 2015). Additionally, application of E2 and the GPER1 agonist, G1, to cultured cortical neurons attenuated NMDA-induced excitotoxicity via activation of MAPK signalling pathways (Liu et al, 2012). These findings demonstrate that activation of GPER1, like mERα and mERß, increases intracellular Ca^{2+} and activates multiple second messenger cascades, suggesting that binding at this receptor could have widespread effects on neuronal function.

1.3 Are there more membrane-associated estrogen receptors to be discovered?

Other potential membrane-associated receptors have been identified. The first is referred to as ER-X. This receptor type is distinguished from ERα, ERß and GPER1 by its molecular weight, which is 63 kDa compared to 67kDa, 60kDa, and 44kDa, respectively (Filardo et al

2007, Toran-Allerand et al 2002). The putative ER-X can also be distinguished from other ERs because the two stereoisomers of E2, 17α- and 17ß- E2 have equal affinity for this receptor, whereas 17ß-E2 has 100 times greater affinity for ERα and ERß than 17α-E2 (Toran-Allerand et al 2002). Another potential membrane-associated ER is a G protein-coupled receptor called Gq-mER that is found in the arcuate nucleus of the hypothalamus and is activated by the selective ER modulator, STX (Qiu et al 2003). STX has a 20-fold greater affinity for this receptor than it does for either ERα or ERβ, and STX has rapid effects in the hypothalamus of GPER1 knockout mice, suggesting that it is binding to an undiscovered ER (Qiu et al 2003). Gq-mER is thought to play an important role in estrogens' effects on metabolic function (Smith et al 2013), and affect multiple homeostatic processes including reproduction, stress, sleep, as well as motivated behaviours (Qiu et al 2008). These potential ERs (i.e. ER-X and Gq-mER) may provide alternate mechanisms via which estrogens can rapidly affect neuronal function, and ultimately cognition. However, there is not sufficient research to speculate on their role in cognition, so the remainder of this review will focus on ERα, ERβ, and GPER1, and their role in dopamine-dependent cognition in females.

2. Distribution of estrogen receptor containing cells in the CNS

Light microscopic immunohistochemical and *in situ* hybridization studies have shown that cells with ERα, ERβ, and GPER1 are found throughout the brain, from the most rostral regions of the forebrain to the cerebellum. It would not be practical to list all regions containing ERs, but regions where high levels of these receptors are consistently observed are described. Interestingly, although these studies observe nuclear labelling for ERα and ERβ throughout the brain, reports of extra-hypothalamic mERα and mERβ using light microscopic methods are limited.

Cells with ERα are most commonly localized to the bed nucleus of the stria terminalis, the medial amygdala, the preoptic area, and various hypothalamic nuclei. High levels of this receptor were also observed in the periaqueductal grey and parabrachial nucleus, and lower levels are observed in the locus coeruleus (Mitra et al 2003, Shughrue et al 1998). The reported distribution of ERβ differs slightly from ERα, but the regions with the most dense labelling are similar, as this receptor has been observed primarily in the lateral septum, the bed nucleus of the stria terminalis, the medial and basolateral amygdala, the preoptic region and other hypothalamic

nuclei, and the trigeminal nuclei (Creutz and Kritzer 2002, Milner et al 2010, Mitra et al 2003, Shughrue et al 1999, Shughrue and Merchenthaler 2001). Intermediate levels of ERβ labelled cells are found in the hippocampus and cerebral cortex (Milner et al 2005, Milner et al 2010, Mitterling et al 2010, Shughrue and Merchenthaler 2001). Some studies have observed ERβ labelled cells in the ventral tegmental area, the locus coeruleus and in granulosa cells of the cerebellum (Mitra et al 2003, Shughrue and Merchenthaler 2001).

GPER1 also is observed throughout the brain, with high levels in the olfactory bulbs, and hypothalamus and various cortical regions including the motor, somatosensory piriform cortices, the hippocampus, and the habenular nucleus of the epithalamus (Brailoiu et al 2007, Hazell et al 2009, Xu et al 2009, Waters et al 2015). More caudally, GPER1 is observed in the nucleus of the solitary tract, and the Purkinje and granule cells of the cerebellum (Hazell et al 2009, Spary et al 2013). Light microscopy observes GPER1 at cytoplasmic sites, and associated with the plasma membrane (Funakoshi et al 2006).

3. Estrogens affect dopamine-dependent diseases and cognitive processes

Estrogens affect a wide array of cognitive processes by altering transmission in various neurotransmitter systems. There is growing evidence that estrogens affect dopamine-dependent cognitive processes. Implications of estrogens' involvement in dopamine dependent diseases comes from clinical observations of sex differences in susceptibility to Parkinson's, schizophrenia, and addiction.

Parkinson's disease is caused by decreased dopamine transmission in the STR, and Parkinson's patients show hippocampal atrophy and decreased markers of neurogenesis in the dentate gyrus (for review see Regensburger, Prots, & Winner 2014). There is a higher incidence of Parkinson's in males (Shulman and Bhat, 2006), however, Parkinson's symptoms in females increase following menopause when endogenous estrogen production decreases (Ragonese et al 2004). Moreover, women respond better to L-3,4-dihydroxyphenylalanine (L-DOPA), the first line treatment for Parkinson's disease, when it is administered with transdermal E2 (Blanchet et al 1999).

Schizophrenia is also hypothesized to result from dysregulated dopamine transmission, with increased dopamine activity in the NAc and STR, and decreased dopamine transmission in the PFC (Howes & Kapur 2009). Individuals with schizophrenia show numerous hippocampal

abnormalities including hippocampal atrophy, and symptom-related changes in hippocampal metabolic activity, among others (for review see Harrison, 2004). Women exhibit later onset and less severe symptomatology than men (Hafner, 2003). However, these symptoms increase when estrogen levels decrease, both during the postpartum period and following menopause (Kulkarni et al 2012, Matevosyan 2011). Moreover, women respond better to antipsychotic drugs when they are administered in conjunction with E2 (Akhondzadeh et al 2003, Kulkarni et al 2014).

Lastly, addiction is also related to dysfunctional dopamine transmission; repeated drug use is associated with a decrease in dopamine release in the STR and NAc (Volkow, Fowler, Wang, Baler, & Telang, 2009), and significant decreases in adult hippocampal neurogenesis leading to changes in the striatal-cortical-frontal circuitry (Chambers, 2013). There are sex differences in the development of addiction, as women escalate use of drugs, including opiates, psychostimulants, and nicotine more rapidly than men (Hernandez-Avila et al 2004, Lynch et al 2002). Women also report a greater response to amphetamines during the luteal phase of the menstrual cycle, when estrogens are high (Justice and de Wit 1999). Collectively, these findings on Parkinson's, schizophrenia, and addiction suggest that estrogens play a role in central dopamine function, as these disorders are all associated with dysfunctional dopamine transmission and hippocampal atrophy/decreased function.

3.1 Estrogens alter dopamine-dependent cognitive processes in rats

There is evidence that estrogens affect many dopamine-dependent cognitive processes, including selective attention object recognition memory and memory system bias. The majority of research has examined the long-term effects of estrogens, administering E2 ~12 hours prior to testing, but research examining the rapid effects of E2 on cognition will be described when available. Latent inhibition (LI), a measure of selective attention, is dependent on dopamine transmission within the mesocorticolimbic pathway. Lesions and local infusion of a dopamine antagonist in the PFC enhance LI (Broersen et al 1996, George et al 2010) as do lesions encompassing the entire NAc (core and shell; Gal, Schiller, and Weiner, 2005), and lesions to the NAc core alone result in abnormally persistent LI (Weiner, Gal, Rawlins and Feldon, 1996). In contrast, lesions of the NAc shell or hippocampus abolish LI (Kaye and Pearce,1987, Oswald et al, 2002: Weiner et al., 2005). Moreover, dopaminergic activity in the anterior STR is positively correlated with behaviour in a LI task (Jeanblanc et al 2003). Interestingly, increases in plasma levels of estrogens, either during the proestrus phase of the estrous cycle or following E2

replacement in ovariectomized (OVX) rats, disrupt the expression of LI (Nofrey et al 2008, Quinlan et al 2010, Almey et al 2013).

Object recognition memory is another dopamine-dependent cognitive process affected by estrogens. Systemic and intra-PFC administration of a D1 antagonist can impair object recognition memory, reflected in poor performance on a novel object preference task (Besheer et al 1999, Nagai et al 2007). Correspondingly, systemic administration of a D1 agonist enhances long-term object recognition memory when administered immediately following training (de Lima et al 2011) or 10 minutes before testing (Hotte et al 2005). Administration of E2 to OVX rats leads to improved object recognition memory (Gervais et al 2013, Jacome et al 2010), an effect that is mimicked by diarylpropionitrile (DPN), an ERβ agonist, suggesting the effects of E2 are at least partially due to binding at ERβ (Inagaki et al 2010, Luine et al 1998). Interestingly, this dose of DPN resulted in a 100% increase in dopamine in the PFC, suggesting that estrogen-induced improvements in recognition memory are due, in part, to increased dopamine (Inagaki et al 2010, Luine et al 1998).

Estrogens also affect the use of place or response memory to navigate an environment. Rats use either spatial or egocentric cues to locate a reward. If spatial cues are used to locate a reward, this is referred to as a place memory, which is mediated by the hippocampus; if egocentric cues are used, this is called a response memory, which is mediated by the dorsal STR (Packard et al 1989, Packard and White 1991, White and McDonald 2002, also see review by Korol and Pisani in this issue). An infusion of amphetamine, an indirect-dopamine agonist, into the hippocampus biases rats toward using place memory, while an infusion of amphetamine into the STR biases rats toward using response memory (Packard and White 1991). Interestingly, when estrogen levels are high, either during the proestrus phase of the estrous cycle or following E2 replacement in OVX females, rats are biased toward using place memory (Korol and Kolo 2002, Quinlan et al 2008). More recently we showed that an infusion of E2 directly into the PFC biases female rats towards use of place memory, indicating that E2 acts rapidly (<15 minutes) to affect memory system bias, possibly via reciprocal projections with the STR and hippocampus (Almey et al 2014). These preclinical studies provide evidence that estrogens affect dopamine-dependent cognitive processes. Some of these cognitive effects of E2 occur rapidly (<4 hours; Almey et al 2014, Gervais et al 2013, Inagaki et al 2010, Jacome et al 2010), suggesting that the

cognitive effects of estrogens result from binding at both nuclear and mERs, leading to long-term and rapid effects.

3.2 Estrogens modulate central dopamine transmission

There is accumulating evidence that estrogens alter dopamine function at various stages in transmission by affecting dopamine availability, dopamine receptor density, and the affinity of the dopamine transporter. Again, the majority of research has examined the long-term effects of estrogens, administering E2 ~12 hours prior to testing, but research examining the rapid effects of E2 on dopamine transmission will be described when available. In the STR, tonic dopamine availability is increased when estrogen levels are high (Xiao and Becker 1994); this increase in dopamine is mediated via both long-term and rapid effect of estrogens, as maximal dopamine increases are observed when E2 is administered ~12 hours prior to testing, and then again 30 minutes prior to testing (Becker and Rudick 1999). When E2 is applied to tissue from the STR this rapidly decreases dopamine uptake by decreasing the affinity of the dopamine transporter (Disshon et al 1998), providing a potential mechanism for E2-induced increases in dopamine availability. Additionally, OVX rats receiving estrogen replacement have higher binding at dopamine D1 and D2 receptors, but lower binding at D3 receptors, in the STR (Landry et al 2002, Le Saux et al 2006, Levesque and Di Paolo 1989, Levesque et al 1989). This increase in dopamine D2 receptor binding occurs without any changes in dopamine D2 mRNA levels (Le Saux et al 2006), indicating that this change occurs through rapid mechanisms.

The relationship between estrogens and dopamine transmission in the NAc is similar to that observed in the STR (Thompson and Moss 1994), as E2 replacement administered to OVX rats is associated with increased tonic (Madularu et al 2014) and phasic (Thompson and Moss 1994) levels of dopamine in the NAc. Systemic administration of E2 increases NAc phasic dopamine release within 15 minutes, indicating this is a rapid effect mediated by mERs (Thompson and Moss 1994). E2 replacement administered to OVX rats was shown to attenuate dopamine reuptake in the NAc (Thompson 1999), providing a potential explanation for the E2-induced dopamine availability in the NAc. Additionally E2 treatment increases dopamine D2 receptor binding in the NAc (Le Saux et al 2006), paralleling findings in the STR.

Estrogens also alter dopamine transmission in the PFC. Females exhibit the highest baseline dopamine levels during estrus, when estrogens are declining, and the lowest basal dopamine levels during proestrus, when estrogen levels are high (Dazzi et al 2007). However,

rats in proestrus have higher ethanol-induced dopamine release in the PFC, suggesting that estrogens decrease basal dopamine, but increase dopamine release in the PFC (Dazzi et al 2007). Additionally, dopamine D1 receptor density as well as dendritic spine density is increased in the PFC when OVX females are treated with E2 (Levesque et al 1989; Wallace et al 2006).

The hippocampus receives a small dopaminergic afferent, and more substantial GABAergic afferents from the VTA (Rocchetti et al., 2015). Additionally, glutamatergic afferents from the hippocampus to the NAc regulate the firing of dopaminergic neurons in the VTA (Floresco, Todd, & Grace, 2001). Moreover, hippocampal projections to the PFC are thought to play a role in amplifying neuronal activity in the PFC (Ishikawa & Nakamura, 2003). These findings suggest that hippocampal afferents can modulate dopamine activity throughout the mesocorticolimbic pathway.

As discussed extensively in previous reviews (McEwen and Alves, 1999; McEwen and Milner, 2007; Spencer et al., 2008; McEwen et al., 2012), the hippocampus is also sensitive to the effects of estrogen. Female rats, either in the proestrus phase of the estrous cycle or following E2 replacement, had significantly elevated spine synapse density in CA1 pyramidal neurons (Gould et al 1990; Woolley et al 1990). The effect of E2 on synaptic spine density in the hippocampus has been shown to occur rapidly, within 30 minutes of subcutaneous E2 administration (MacLusky et al 2005). *In vivo* studies demonstrate that female rats administered an infusion of E2 into the hippocampus demonstrate better recollection for the platform location in a watermaze task (Packard and Teather 1997), and demonstrate a bias towards use of place memory to navigate a maze (Zurkovsky et al 2007).

Although estrogens have been shown to affect dopamine-dependent cognitive processes in the STR, PFC and hippocampus, these brain are not recognized for having high levels of ERs. Generally, light microscopy and *in situ* hybridization observe low levels of ERα immunoreactivity (IR) in the STR and NAc, and almost none in the PFC (Mitra et al 2003, Shughrue et al 1998; see also figures 2 & 4), although other light microscopy studies report moderate levels of ERα in the PFC (Montague et al, 2008). Light microscopy studies observe ERβ–IR at low levels in the PFC, and very low levels in the STR and NAc (Mitra et al 2003, Milner et al 2010; see also figures 2 & 4). Similarly, *in situ* hybridization studies observe low levels of ERβ in the STR and NAc, and extremely low levels of the receptor in the PFC (Shughrue et al 1999). Moderate levels of ERα-IR profiles were observed in interneurons of the

hippocampus using light microscopy, with the highest density in the stratum radiatum and dentate hilus (Solum and Hanada 2001, Weiland et al 1997), and ERβ-IR is observed throughout the hippocampus at moderate levels (Zhang et al 2002). The immunolabelling for ERα and ERβ observed in these light microscopy experiments was primarily nuclear labeling (Mitra et al 2003, Zhang et al 2002); these nuclear receptors are likely responsible for the long-term actions of estrogens in these regions.

However, there are rapid behavioural effects of estrogen in the STR, NAc, PFC and hippocampus (Almey et al 2014, Cornil and Charlier 2010, MacLusky et al 2005, Thompson 1999, Thompson and Moss 1994), which could not occur through binding nuclear ERs. Immunolabelling for GPER1 is observed at relatively high levels in the hippocampus, moderate levels in the PFC, and lower levels in the STR and NAc (Hazell et al 2009), presumably at the cell membrane or associated with cytoplasmic organelles (Almey et al 2012, Otto et al 2008). Therefore, binding at GPER1 could be responsible for some of the rapid effects of E2 in these brain regions, but the rapid effects of E2 also suggest mERα or mERβ may be present as well. Some light microscopy studies observe non-neuronal mER-IR profiles (Milner et al 2005, Wagner, Silverman, and Morell 1998, Zhang et al 2002), but the majority do not (Almey et al 2014, Almey et al 2013, Cruetz and Kritzer 2002, Mitra et al 2003, Shughrue et al 1998, Weiland et al 1997), which suggests that light microscopy does not have sufficient resolution to detect low levels of mERs. Immunoelectron microscopy methods have higher resolution than light microscopy (See Box 1), and can discriminate discreet labeling for mERs in the brain (Milner 2011). Consequently, this technique was used to clarify the subcellular localization of mERs to specific neuronal profiles in the hippocampus. The effects of estrogen on cognition likely result from a combination of long-term and rapid actions; there is an impetus to clarify the distribution of mERs, to better understand this rapid component of estrogen's effects.

4. Ultrastructural localization of ERs in the hippocampus

The earliest incidence of ultrastructural localization of ERs examined the distribution of ER (later called ERα) in hypothalamic tissue (Blaustein et al 1992, Langub and Watson 1992). Landub and Watson (1992) focussed on nuclear labeling for ERα in the medial preoptic area and the median eminence. While examining the ultrastructural localization of nuclear ERα in the hypothalamus, a second research group noticed that ERα-IR profiles were also localized to

extranuclear sites in both dendrites and terminals (Blaustein et al 1992). These findings implied that estrogens could have rapid effects on transmission in the hypothalamus, in agreement with the observation that increases in estrogens during proestrus reduced gonadotropin releasing hormone and luteinizing hormone in 30 minutes, an effect so rapid that it could not be elicited via cytoplasmic ERs (Condon et al 1988, Sarkar and Fink 1980). This initial electron microscopic research in the hypothalamus was not pursued, despite that it provided a mechanism for the rapid effects of estrogens in this region. However it did suggest that electron microscopy is a powerful tool for observing mERs in the brain. Ultrastructural analysis of the distribution of ERs recommenced in the hippocampus in the late 90s, based on evidence from McEwen and colleagues demonstrating that estrogens induce rapid structural changes in the hippocampus. mERα was localized to the cytoplasmic surface of the membrane in interneurons throughout the hippocampus, with the highest density in the stratum radiatum of the CA1 (see Fig 2B; Milner et al 2001). In the hippocampus, 50% of all mERα-IR profiles are presynaptic profiles, i.e. axons and terminals. Presynaptic terminals with mERα-IR form both asymmetric and symmetric synapses, suggesting that estrogens affect both excitatory and inhibitory transmission in the hippocampus (Milner et al 2001). Additionally 25% of the total mERα-IR profiles are post-synaptic, i.e. dendrites and dendritic spines (Milner et al 2001), providing a potential mechanism for the E2-induced changes in synaptic spine density observed in the hippocampus (Gould et al 1990). The remaining 25% of mERα-IR profiles in the hippocampus are glial, including astrocytes and microglia (Milner et al 2001), suggesting a mechanism for E2 involvement in glial-mediated neuroprotection (Arevalo et al 2010). After this initial study in rats, subsequent studies have shown that ERα-IR profiles in the mouse hippocampus are similarly distributed (Mitterling et al 2010). Some of the mERα-IR profiles observed in the hippocampus are localized to cholinergic axons and terminals, identified via the vesicular acetylcholine transporter (VAChT), which suggests that mERα is positioned to have rapid effects on cholinergic transmission in this region (Towart et al 2003). Additional electron microscopic autoradiography studies have shown that
125I-E2 binds to both pre- and post-synaptic profiles in the hippocampus supporting a functional role for nonnuclear ERs (Milner et al 2008).

Ultrastructural studies have demonstrated that mERα is associated with small synaptic vesicles in a subset of GABAergic axons and terminals in the CA1 of the hippocampus; findings

from this experiment suggest that estrogens may bind at these receptors to mobilize vesicles towards synapses (Hart et al 2007). Further research demonstrated that some of the mERα-IR vesicles contain GABA (Tabatadze et al 2013), and that E2 acts via ERα to reduce GABA release through interactions with the cannabinoid system (Huang and Woolley 2012). Together these findings demonstrate that mERα is observed in the hippocampus, where it is positioned to affect presynaptic transmission in GABAergic and cholinergic neurons, and affect E2-induced increases in spine density.

The success of these studies on mERα distribution in the hippocampus led to studies examining the distribution of mERβ and GPER1 in the rodent hippocampus. mERβ-IR was also observed at extranuclear sites in the hippocampus, in the CA1, CA3, and dentate gyrus (see Fig 2A; Milner et al 2005, Mitterling et al 2010). Profiles with mERβ-IR were primarily post-synaptic: ~25% of the total mERβ-IR profiles were dendritic shafts, and ~15% of the mERβ-IR profiles were dendritic spines. There was a greater percentage of mERβ localized to dendritic spines in the CA1 region of the hippocampus (Milner et al 2005), where E2-induced changes in spine synaptic density are observed (Gould et al 1990). Ten percent of all mERβ-IR profiles were axon terminals, and 20% of the mERβ-IR profiles were axons, suggesting that binding at mERβ would also affect presynaptic transmission in the hippocampus. Similar to mERα, mERβ-IR was localized to endomembranes, including the membranes of endoplasmic reticulum and mitochondria, and has been observed in glial cells in the hippocampus (Milner et al 2005). See earlier reviews (McEwen and Alves, 1999; McEwen and Milner, 2007; Spencer et al., 2008; McEwen et al., 2012) for an in-depth discussion of estrogen effects in the hippocampus.

Following the discovery and characterization of the newest ER, GPER1, light microscopic studies observed GPER1-IR in several subregions of the rodent hippocampus (Brailoiu et al 2007, Funakoshi et al 2006). Moreover, electron microscopic studies observed GPER1-IR at the plasma membrane of pyramidal cells in the CA2 (Funakoshi et al 2006). Recently, collaborative studies with the Milner lab have furthered these findings, demonstrating that GPER1-IR is localized to pre- and post-synaptic sites in both the rat and mouse hippocampus (see Fig 2C; Akama et al 2013, Waters 2015). Notably, GPER1 in this region is exclusively extranuclear, found in pyramidal cells and interneurons throughout the hippocampus (Waters 2015). Within perikarya, GPER1-IR is affiliated with the plasma membrane and

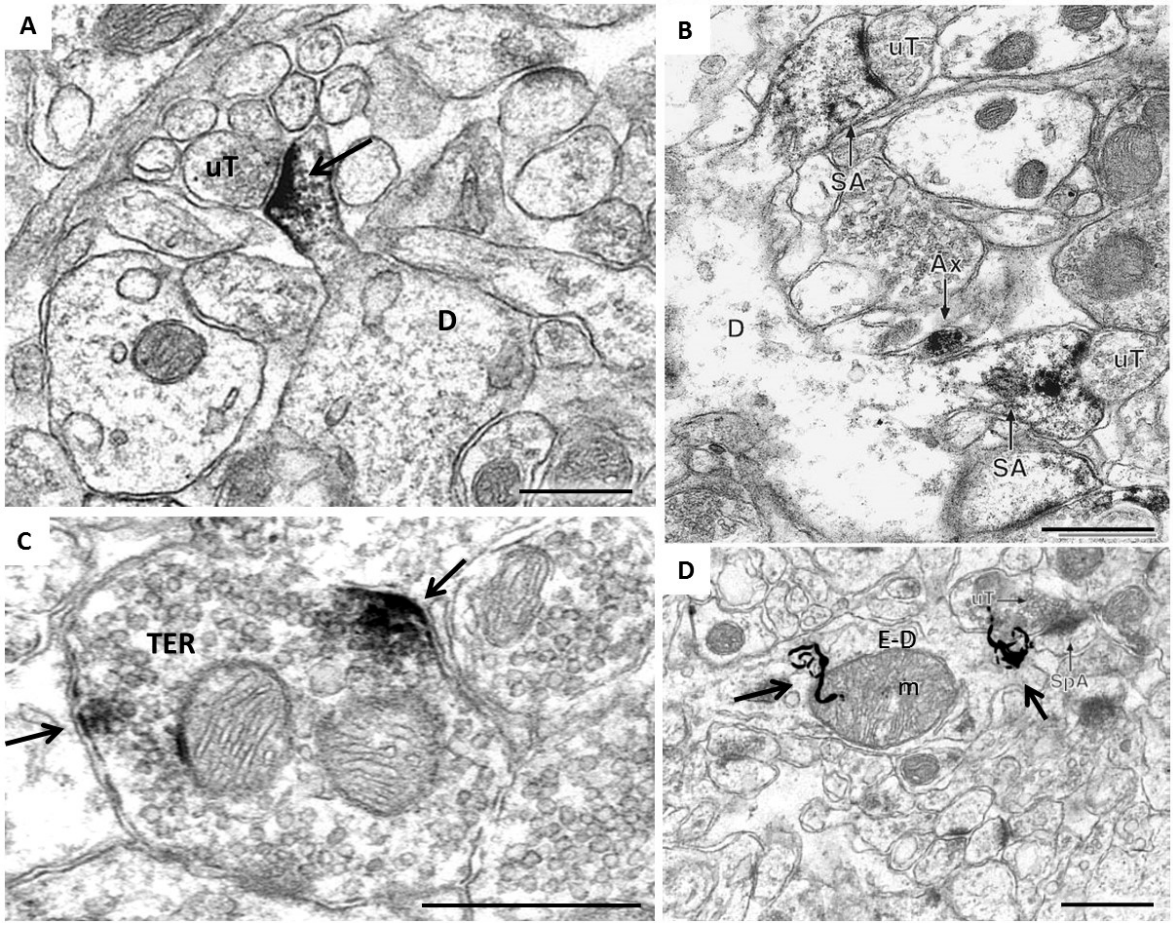

Figure 2. Images of immunomarkers for estrogen receptors and estradiol in the hippocampus of female rats. **A)** Immunoperoxidase labeling for ERβ in a dendritic spine is contacted by an unlabeled terminal (uT) in CA1 stratum radiatum, **B)** Immunoperoxidase labeling for ERα is found in two dendritic spines identifiable by the presence of spine apparati (SA), which arise from the same dendrite (D). Both labeled spines are contacted by unlabeled terminals (uT), and an ERα-labeled axon (Ax) is found nearby, **C)** Clusters of immunoperoxidase labeling for GPER1 are found in small synaptic vesicles near the plasma membrane of a terminal, **D)** Autoradiographic silver grains (*black squiggly lines*) denoting ^{125}I-estradiol binding in stratum radiatum of the CA1 region of the hippocampus in a dendrite (E-D) overlying a mitochondrion (m). *Black arrows* = peroxidase/radioactive marker, *Scale Bar* = 500nm.

endoplasmic reticulum. Like mERα and mERβ, GPER1-IR is localized to dendritic spines, suggesting that estrogens could alter dendritic spine morphology by binding at GPER1. Moreover, GPER1 is found in axons and clusters of vesicles in axon terminals, especially in CA3, where it could regulate synaptic transmission (Waters et al 2015).

Together, electron microscopic studies have demonstrated that profiles containing mERα, mERβ and GPER1 are abundant in the hippocampus. Light and electron microscopic studies also have revealed that ERs are in hippocampal neurons that undergo adult neurogenesis which are known to be important in cognitive processes (for review see Leuner et al 2006). Systemic administration of estrogens significantly increases cell proliferation in the dentate gyrus in a dose-dependent manner (Gould et al 2000, Tanapat et al 2005). Using *in situ* hybridization, Isgor and Watson (2005) demonstrated that ERα and ERβ mRNA are expressed in new cells in the hippocampus. Electron microscopic studies have revealed that newly generated cells in the subgranular region of the dentate gyrus express mERβ-IR at the plasmalemmal membrane and the membrane of cellular organelles (Herrick et al 2006). These findings implicate estrogens in the genesis, and/or maturation of cells in the hippocampus.

Additionally, estrogens have been shown to increase production of pre- and post-synaptic proteins in the dorsal region of the CA1 (Brake et al 2001), an effect that is mediated by both ERα and ERβ (Spencer-Segal et al 2012, Waters et al 2009). This provides further evidence that estrogens are involved in synaptogenesis in the hippocampus, suggesting that estrogens play a role in the formation of new synaptic connections. It has also been shown that systemic E2 administration results an increase in phosphorylated Akt 6 hours following administration, and an increase in phosphorylated TrkB 48 hours following administration—an effect that occurs via binding at both ERα and ERβ in the hippocampus (Spencer-Segal et al 2012, Spencer et al 2008). Moreover, electron microscopic studies have shown that estrogens regulate the levels and trafficking of pAkt and pTrkB in hippocampal neurons (Spencer-Segal et al 2011, Yildirim et al 2011, Znamensky et al 2003). Both of these signalling pathways are implicated in synaptic plasticity, so E2-induced activation of these pathways provides another mechanism through which estrogens may cause synaptic strengthening or remodelling. Current theories on the neural mechanisms responsible for memory formation postulate that synaptic plasticity, synaptogenesis, and neurogenesis work in concert in the hippocampus, allowing for memory formation and retention. Therefore, the estrogen-induced changes in these brain plasticity mechanisms could

impact hippocampal-dependent memory. Interestingly, post-embedding electron microscopic studies have revealed that aging negatively affects trafficking of mERα (Adams et al 2002) and mERβ (Waters et al 2011) in synapses within the hippocampus; together these findings provide a potential mechanism for the cognitive impairments observed in post-menopausal women.

The success of ultrastructural analysis in the hippocampus led to experiments examining the distribution of mERs in other brain regions where estrogens are known to have effects, despite a paucity of nuclear receptor staining. Prior reviews have discussed the localization of ERs in autonomic circuits (McEwen et al 2012), so it is not discussed here. Additionally, ultrastructural analysis revealed that there was extranuclear mERα observed in serotonergic neurons of the raphe nuclei (Milner 2003), providing a mechanism for rapid effects of estrogens on serotonergic transmission in this region.

Rationale and Hypotheses:

It is clear that estrogens affect several cognitive processes, including selective attention, reversal learning, multiple memory systems, and object recognition learning, among others. This thesis endeavored to explore the cognitive effects of estrogens in female rats, as well as examining the effects of estrogens in the context of schizophrenia, using rodent models of this disorder. The effects of estrogens on cognitive deficits observed in schizophrenia, and the response to the antipsychotic drug haloperidol (HAL) were assessed in female rats. To accurately assess the effects of estrogens on these cognitive processes naturally cycling estrogens were eliminated by ovariectomizing all female rats in the behavioural experiments. These rats were administered no estrogen replacement, a low chronic dose of 17β E2 mimicking plasma levels of E2 during the diestrus phase of the cycle, or a high cyclic dose of E2 that mimicking plasma levels of E2 during the proestrus phase of the cycle.

Previous research in this lab has demonstrated female rats with high plasma levels of E2, following estrogen replacement or in the proestrus phase of the cycle, during the conditioning phase of LI exhibited attenuated LI. Chapter 2 extends these findings, examining the effects of no, low and high E2 replacement administered alone, or in conjunction with an acute dose of HAL, on behaviour of male and female rats in a LI task. These experiments attempted to confirm previous results with female rats, which showed that estrogens have detrimental effects on selective attention, and asked whether LI in male rats paralleled LI in females with or without estrogens. Additionally these experiments examined whether there was an interaction between the E2 replacement regimen and HAL treatment. It was hypothesized that higher E2 replacement would be associated with attenuated LI compared to female rats that received no E2 replacement and males. Additionally, it was hypothesized that E2 would facilitate the antipsychotic drug HAL to restore LI. To investigate these questions female and male rats were run on a conditioned emotional response LI procedure in operant chambers. Female rats were OVX and administered no, low, or high E2 replacement to determine whether plasma levels of E2 affected selective attention, as measured by the LI paradigm. Additionally female and male rats were administered saline, 0.05mg/kg or 0.1mg/kg acute doses of the antipsychotic HAL to determine how HAL affected selective attention and whether there was an interaction between E2 and HAL.

Following the LI experiments, Chapter 3 extended these findings to two other cognitive processes. These experiments examined the effects of the same E2 replacement regimens (no, low, and high E2), administered alone and in conjunction with HAL, on perseveration and reversal learning. These experiments asked whether E2 affected perseveration or reversal learning in female rats, and whether there was any interaction between the E2 replacement and a chronic HAL treatment. The methodology was modified for these experiments to increase their ecological validity by using an animal model of schizophrenia; prior to behavioural testing all rats were amphetamine sensitized, since this induces some of the cognitive symptoms of schizophrenia, including increased perseveration and deficits in reversal learning. It was hypothesized that E2 would have a detrimental effect on these cognitive processes, increasing perseveration and the latency to reach reversal learning criterion, but that E2 would facilitate HAL to reduce perseveration and improve reversal learning. To address these questions female rats were trained to press a lever in an operant chamber to receive sucrose reinforcement. Perseveration was measured as the time it took to extinguish lever pressing behaviour, and reversal learnin was measured as the time it took for rats to consistently switch from pressing the formerly active lever to the newly activated lever. All rats were OVX and administered either no, low or high E2 replacement; half of the rats in each hormone replacement group were administered a chronic dose of HAL and the other half were administered saline. The different hormone and drug treatment groups were compared on their perseveration and reversal learning behaviour.

There is evidence that LI, perseveration, and reversal learning are all dopamine dependent disorders, mediated in part by the STR, NAc, and PFC (Castane, Theobald, & Robbins, 2010; Ersche et al., 2011; Gal et al 1997, Jeanblanc et al, 2003; Nelson, Thur, Marsden, & Cassaday, 2010; Oswald et al, 2002; Piantadosi & Floresco, 2014; Schiller & Weiner, 2004; Taghzouti, Louilot, Herman, Le Moal, and Simon, 1985). Furthermore, there is evidence that infusions of estrogen directly into the STR rapidly (<2hrs) disrupt response memory (Zurkovsky et al, 2011), systemic injections of estrogens rapidly (<30min) increase dopamine availability in the STR, and infusions of E2 into the NAc rapidly altered dopamine transmission in this region (Thompson and Moss, 1994;1997). Consequently it was posulated that estrogens elicited their effects on these behaviours and the response to HAL by affecting dopamine transmission, potentially in the STR, NAc, or PFC. The mechanism by which estrogens could affect dopamine

availability and response memory were unclear, since light microscopy and experiments reported almost no nuclear labeling for ERα and ERβ in the STR, and low levels in the NAc. Since the effects of E2 in these regions occurred rapidly, it was hypothesized that ERs were localized to extranuclear sites. Electron microscopy was successfully used to visualized mERs in the hippocampus, so the next series of experiments used this technique to examine the ultrastructural distribution of mERs in the STR, NAc and PFC.

The experiments in Chapter 4 examined the distribution of ERs, ERα, ERβ, and GPER1, in the STR. Chapter 4A was a study that asked whether ERα, ERβ, and GPER1 were localized to the STR, and whether these receptors were observed on dopaminergic or cholinergic neurons. Chapter 4B confirmed the ultrastructural distribution of ERα and GPER1 in the STR, and examined whether ERs were localized to GABAergic neurons in the STR. It was hypothesized that mERα, mERβ, and GPER1 would be observed in the STR, and that some of these mERs would be localized to dopaminergic neurons. Additionally, it was also hypothesized that a proportion of mERs were localized to cholinergic and GABAergic neurons in the STR.

Following the experiments in the STR, similar experiments were conducted examining the distribution of ERα, ERβ, and GPER1 in the Core and Shell subregions of the NAc, described in Chapter 5. These experiments examined whether ERα, ERβ, and GPER1 were observed in the NAC, and whether these mERs were localised to dopaminergic neurons, since estrogens in the NAc rapidly affect dopamine transmission (Thompson and Moss, 1994). Additionally, based on findings in the STR, an experiment also examined whether ERα and GPER1 were localized to GABAergic neurons in the NAc Core and Shell. It was hypothesized that membrane associated estrogens receptors would be localized to the NAc, potentially on catecholaminergic neurons, since there are rapid effects of estrogens on dopamine transmission in this region (Thompson and Moss, 1994; 1997), and also on GABAergic neurons. Again, electron microscopy and immunolabelling were used to visualize ERs in the NAc, and determine what type of neurons these receptors were localized to. All analyses were run separately in the NAc Core and Shell to determine if the distribution of ERs differed between these two subregions of the NAc.

Following the success of the electron microscopy experiments examining the distribution of ERs in the STR and NAc, the distribution of these receptors in the PFC was then examned. Additionally, research has primarily examined the long-term effects of estrogens on cognition; it

remained unclear whether estrogens could elicit rapid effects on cognition through binding at mERs. The final chapter of the thesis, Chapter 6, contains two experiments. The first experiment examined whether mERs were observed in the PFC, and the second experiment asked whether binding at mERs in the PFC could rapidly alter cognition, specifically multiple memory systems. Based on the results of Chapters 4 and 5, which observed abundant mERs in the STR and NAc, it was hypothesized that ERα, ERβ, and GPER1 would also be observed at extranuclear sites in the PFC. To address this questions, electron microscopy and immunolabelling techniques were used again to examine the distribution of ERs in the PFC. For the experiment on multiple memory systems, there is evidence that higher plasma levels of estrogens bias female rats towards use of a place strategy, so it was hypothesized that E2 in the PFC would rapidly bias rats towards use of place memory to navigate in their environment. To assess the effects of estrogens on memory system bias female rats were administered an infusion of E2 into the medial PFC, and memory system bias was assessed with a t-maze task.

CHAPTER 2:
DEFICITS IN LATENT INHIBITION INDUCED BY ESTRADIOL REPLACEMENT ARE AMELIORATED BY HALOPERIDOL TREATMENT

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Abstract:

There are sex differences in the symptomatology of schizophrenia, and in the response to antipsychotic treatments. One hallmark symptom of schizophrenia is a deficit in selective attention. Selective attention can be measured using an LI paradigm in humans; LI can be measured in rodents, and is used as an animal model of the selective attention deficits observed in schizophrenia. In the current experiments LI was used to clarify whether selective attention differs between male rats and OVX female rats receiving different E2 replacement regimens. An additional aim was to determine whether HAL's facilitation of LI is enhanced by E2. Males and OVX female rats were trained in a conditioned emotional response LI paradigm. Females received no E2 replacement, a chronic low dose of E2 via silastic capsule, or a high phasic dose of E2 via silastic capsule accompanied by E2 (10 μg/kg SC) injections every fourth day. Actual plasma levels of E2 were determined using an enzyme linked immunosorbent assay. Rats were also administered a vehicle treatment, a 0.05mg/kg, or a 0.1mg/kg IP injection of HAL. Males and OVX females that did not receive E2 replacement both exhibited LI, but LI was not observed in the low and high E2 replacement groups. HAL restored LI at a lower dose in the females receiving high E2 replacement compared to females receiving low E2 replacement, indicating that E2 replacement facilitates HAL in restoring LI.

1. Introduction

There are sex differences in schizophrenia, particularly in the positive symptoms of this disorder, with women developing schizophrenia later in life and exhibiting less severe symptomatology than males (Häfner, 2003; Häfner et al., 1992; Kulkarni et al., 2008). It has been suggested that estrogens are, in part, responsible for these sex differences, reducing the frequency of hospital admissions and diminishing the psychotic symptoms associated with schizophrenia (Kendell et al., 1987; Matevosyan, 2011). Additionally, there is some evidence that estrogens are linked to greater efficacy of antipsychotic treatments, as positive symptoms of schizophrenia are further reduced when antipsychotics are co-administered with estrogen than when administered alone (Akhondzadeh et al, 2003; Seeman, 2004). These studies suggest that estrogens protect against the positive symptoms of schizophrenia and facilitate antipsychotic treatments in ameliorating these positive symptoms. However, it remains unclear whether estrogens also have these effects on the cognitive symptoms of schizophrenia.

One specific cognitive deficit associated with schizophrenia is disrupted selective attention, which is often assessed using an LI paradigm (Escobar et al, 2002). With LI, non-reinforced preexposures to a stimulus impair subsequent conditioning, as these preexposures result in reduced allocation of attention to that stimulus (Lubow, 1989). However, individuals with acute schizophrenia and schizoptypy have impaired performance in LI, as repeated non-reinforced preexposures to a stimulus do not retard conditioning to that stimulus (Kaplan and Lubow, 2011; Lubow et al, 2000; Schmidt-Hansen et al, 2009). Individuals with chronic, medicated, schizophrenia do not exhibit disrupted LI, indicating that antipsychotic treatment ameliorates this deficit in selective attention (Gray, 1998). It has been argued that this attentional deficit in schizophrenics, characterized by the processing of irrelevant stimuli, is a critical contributor to the symptomatology of this disease (Brébion et al, 1996; Schmidt-Hansen et al, 2009). Although deficits in selective attention are a hallmark of schizophrenia, few studies have examined whether there are sex differences in this cognitive symptom, and how ovarian hormones may contribute to such differences. Preliminary research in both schizophrenic and non-schizophrenic populations, indicates that males exhibit LI under conditions where females do not (Vol'f et al, 2001; Kaplan and Lubow, 2011). The reason for these sex differences is currently unknown, but it is possible that higher levels of estrogens in females may contribute to this deficit in selective attention. Additionally, antipsychotic treatments ameliorate the

deficiencies in selective attention observed in schizophrenics, but it remains unclear whether there are sex differences in this response to antipsychotic medication.

LI can be assessed in both rodents and humans, and LI paradigms are used to model the attentional deficits associated with schizophrenia in animals (Lubow, 2005). Repeated psychostimulant treatment, shown to induce a psychotic state in humans (Lichlyter et al, 2011), abolishes LI in rats (McAllister, 1997; Moran et al, 1996; Ruob et al, 1997). Administration of antipsychotics, including HAL (Arad and Weiner, 2009; Dunn et al, 1993; Feldon and Weiner, 1991; Ruob et al, 1997; Weiner et al, 1997), among others (Arad and Weiner, 2009; Moran et al, 1996), facilitate LI in male and female rats in a dose-dependent manner. These antipsychotics also recover LI following a chronic psychostimulant treatment that abolishes LI (Moser et al, 2000), demonstrating that antipsychotics have similar effects on behaviour in the LI paradigm in rats and humans.

Experiments examining the effect of estrogens on behaviour of rats in an LI paradigm have yielded contradictory results. Research from our lab and others indicates that high circulating levels of estrogens on the conditioning day of an LI paradigm, either during the proestrus phase of the estrous cycle or in response to E2 replacement following ovariectomy, are associated with disrupted LI (Arad and Weiner, 2008; Nofrey et al, 2008; Quinlan et al, 2010). Correspondingly, low levels of circulating estrogens on the conditioning day of the same LI paradigm, either during the estrus phase of the estrous cycle or following ovariectomy, are associated with intact LI (Arad and Weiner, 2008; Nofrey et al, 2008; Quinlan et al, 2010). This suggests that estrogens may be detrimental to selective attention, reflected in the disruption of LI observed when circulating levels of estrogens are high. Recent research in humans used a paradigm that elicited LI in males, but not females (Vol'f et al, 2001; Kaplan and Lubow, 2011), which suggests that estrogens may also have detrimental effects on LI in humans. In contrast to these findings, it has also been shown that elimination of circulating estrogens via ovariectomy disrupts LI, and estrogen replacement following ovariectomy eliminates this deficit in LI (Arad and Weiner, 2009; 2010A; 2010B). This would suggest that estrogens facilitate, not disrupt, selective attention. Research is needed to clarify the effect of estrogens on behaviour observed in the LI paradigm.

The current experiments investigated the effects of E2 on selective attention, and the combined effects of both E2 and HAL on selective attention. In the first experiment, the

behaviour of males and OVX females receiving no E2 replacement, a low chronic E2 replacement, or a high phasic E2 replacement, were compared in a conditioned emotional response LI paradigm. This experiment also administered two doses of the antipsychotic, HAL (0.05 and 0.1mg/kg), to determine, in a dose dependent manner, if the effects of HAL on LI varied based on sex or E2 replacement. In the first experiment, the aim was to use E2 replacement doses that mimic levels of E2 observed during the diestrus and proestrus phases of the estrous cycle. To ensure that E2 replacement doses achieved the desired levels of circulating E2, a second study was conducted. That study examined plasma levels of E2 in OVX rats administered the same E2 regimens as study 1 to quantify the plasma concentration of E2 over time following the E2 replacement regimes used here.

2. Methods

2.1 Subjects and Surgeries

Subjects included 58 male and 179 OVX female Sprague–Dawley rats weighing approximately 260g or 240 g, respectively, at the beginning of the experiment (Charles River Laboratories, St Constant, QC). Rats were housed in shoebox cages in a colony room maintained on a reversed 12:12 h light/dark cycle (lights off at 08:00 h) at approximately 21 °C. Prior to surgery the animals were housed in pairs (but separated by sex), and following surgery animals were individually housed. All animals were handled daily, except during recovery from surgery. Food was available ad libitum throughout the experiment. Water was available ad libitum until a day before the start of the experiment, at which time water bottles were removed from cages and replaced for 30 min 2 h after the end of the daily experimental session. All animal handling and testing procedures were approved by the Animal Research Ethics Committee (AREC) of Concordia University, and were in accordance with guidelines established by the Canadian Council on Animal Care.

Approximately 4-7 days after arrival all animals underwent surgery. Females were ovariectomized bilaterally through a lumbar incision, and males received sham surgeries, under isofluorane gas anaesthetic (4% for induction, 2% for maintenance) using aseptic procedures. Post-surgical care included administration of the analgesic Anafen (0.1ml, SC), the antibiotic penicillin (0.1ml, intramuscular), and physiological saline to prevent dehydration (3ml, SC). All rats received a week-long recovery period prior to the start of the experiments.

2.2 Drug and Hormone Treatments

Female rats were assigned to one of three hormone treatment groups: no E2 replacement, low chronic E2 replacement, or high phasic E2 replacement. At the time of OVX surgery rats in both the low and high E2 groups were implanted subcutaneously with a silastic capsule containing 5% 17-β E2 (Sigma-Aldrich, St. Louis, MO) in cholesterol (Sigma). These capsules have been reported to produce a consistent serum concentration of E2 of ~20pg/ml (Mannino et al, 2005), which is within the range observed in the diestrus phase of the estrous cycle (Overpeck et al, 1978). Rats in the high phasic E2 treatment group received a subcutaneous injection of E2 (10ug/kg, SC) dissolved in sesame oil every fourth day to mimic levels of E2 observed during the proestrus phase of the cycle. All other rats received oil vehicle injections at this time. Injections of E2 or vehicle were administered after the session in the operant chambers on day 3 and 7 of the experiment. E2 was administered on day 7 so rats would be exposed to E2 approximately 16 hours prior to the conditioning session on day 8 of the experiment.

HAL (diluted in 0.9% saline; Sandoz Inc, QC, Canada) was administered at 0, 0.05 or 0.1 mg/kg, IP. Doses were selected following a dose-response pilot study. A pilot study initially used a range of doses up to 0.2 mg/kg IP of HAL, corresponding to typical doses administered to males (Moser et al, 2000) and females (Arad and Weiner, 2009). However, this higher dose induced sluggishness and torpor in the high E2 female rats, evidenced by a lack of voluntary movement and diminished reaction to the footshock. Consequently, the HAL treatments were restricted to 0.05mg/kg and 0.1 mg/kg. HAL injections were administered to the rats on the morning of day 8 of the experiment ~45min before the beginning of the conditioning session.

2.3 Latent Inhibition

Modular operant test chambers (25 cm wide × 30 cm long × 30 cm high) contained within sound-attenuating isolation units were used for all behavioural training and testing (Coulbourne Instruments, Allentown, PA). Each chamber was equipped with a center house light (2.8 W) 27 cm above the grid floor in the center of the left hand wall of the box. A speaker was located just below the house light, and the grid floor was connected to a shock module. A water lickometer was located across from the house light approximately 8 cm above the floor. Every time an animal licked the water bottle positioned behind the lickometer an infrared beam was interrupted; the number of infrared beam interruptions was used as the measure of drinking

behaviour. Conditioning chambers and data acquisition were controlled by a desktop personal computer running Graphic State Notation software (Coulbourne Instruments, Allentown, PA).

Water restriction began the afternoon prior to the beginning of behavioural testing, at which point water bottles were removed from the home cages. The following morning rats were placed in the operant test chambers and allowed access to the water for 20min; this habituation continued for the first 6 days of the experiment to guarantee that the rats learned to drink water in the chamber. Rats that did not learn to drink water in the operant boxes sufficiently (100 licks per session) were omitted from the study. The number of licks per session was recorded as baseline drinking behaviour. Following habituation, on day 7 of the experiment, water bottles were removed from the operant chambers. Half of the animals from each group were presented with 40, 5sec, 2.5 kHz tones at a volume of ~65 decibels (Preexposed group, PE). The 40 tone presentations were presented at 10-50s variable intervals over a 22.5 min session in the operant chambers. The other half of the animals were placed in the test chambers for an equivalent amount of time but were not presented with the tone (Not Preexposed group, NPE). On day 8 all rats underwent a conditioning session where they were subjected to two pairings of the 5sec tone directly followed by a 0.5 mA foot shock for a duration of 1sec. The inter-shock interval was five min. On the 9th day the water bottles were returned to the boxes and animals were re-habituated to drinking in the chambers.

Testing occurred on the 10th day when water bottles were present in the operant boxes. After the rats made 100 licks from the water bottle the tone turned on and remained on until the rat made 20 additional licks or until 5 min elapsed. This allowed for measurement of drinking behaviour both with and without the tone. In this paradigm it is hypothesized that since the tone was previously paired with the footshock, then subsequent presentation of the tone should inhibit drinking. The inhibition of drinking behaviour is expected to be greater in rats in the NPE group, who were only presented with the tone paired with a footshock. Conversely, the PE group who heard the tone 40 times without any consequence should not associate the tone and footshock as strongly, and their drinking should be less inhibited. This paradigm is thought to indirectly measure freezing behaviour, since the decrease in drinking following the onset of the tone is typically the result of the rat freezing, a characteristic fear response (Sotty et al, 1996). LI is considered to have occurred when the rats in the PE group make 20 licks with the tone on faster than rats in the NPE group.

2.4 E2 Plasma Level Assessment

A separate study was conducted to assess the plasma level accuracy of the E2 treatments used in the behavioural experiment. To examine the efficacy of the silastic capsule alone, E2 plasma concentrations were examined for one month following its implantation. This experiment included five female Sprague Dawley rats that were OVX by a lumbar incision in the exact manner as described for the previous experiment (see 2.2). One week post ovariectomy, rats were implanted with a silastic capsule containing 5% E2 as described in the previous study. Blood was collected from the tail vein of these rats one week following ovariectomy, prior to E2 implant, and again at 1, 2, 3, and 4 weeks following capsule implantation.

In addition, to examine the plasma level effects of an acute E2 injection, seventeen female Sprague Dawley rats were OVX by a lumbar incision and implanted with an E2 containing silastic capsule as described previously. Ten days following the surgery all rats were administered a 10µg/kg IP injection, of E2 dissolved in sesame oil (see 2.2). E2 was assayed in sera from rats at six time points across a 24hr period; any one rat was only used for two time points for ethical reasons. Blood was collected from the tail vein of five rats just prior to the injection (baseline), and then again 4 hrs following the injection. Blood was collected from six different rats at 8hrs and again at 12hrs following the injection. Finally, blood was collected from the final six rats at 16hrs and again at 20hrs following injection.

Blood was collected in ice-cold vials and immediately centrifuged. Plasma was stored at -20°C until it was assayed for E2 using a commercially available ELISA kit (Immuno-Biological Laboratories Inc., Minneapolis, MI). The assay antibodies have 100% cross-reactivity with E2 and 0.2% and 0.05% cross-reactivity with estrone and estriol, respectively. The range of the assay is between 0 and 2000 pg/ml and the reported inter-assay variation is 7–9%.

2.5 Statistical Analyses

In an LI protocol the suppression ratios in the PE and NPE groups are compared to determine if LI has occurred in the PE group. For each rat, a suppression ratio was calculated as a measure of LI. This was measured as the time to complete licks 81–100[A – without the tone] divided by the sum of the time to complete licks 81–100 and licks 101–120[B – with the tone] (A/A + B). Here, a suppression ratio of 0.5 indicates no suppression of licking during the tone (i.e., no conditioned response to the tone), while a suppression ratio of 0.003 indicates full suppression of licking in the presence of the tone. Suppression ratios for the rats in each group

were averaged; LI is considered to have occurred when the rats in the PE group have significantly higher suppression ratio than that in those in the NPE group.

Data from the males and females were analyzed separately, as hormone treatment level was a factor in the analyses for females but not for the males. Suppression ratios from the males were analyzed using a 2x3 between subjects ANOVA, and suppression ratios for the females were analyzed using a 2x3x3 between subjects ANOVA. Additionally, planned orthogonal contrasts were run on all of the data, comparing the PE and NPE groups in each condition to determine if LI occurred. T-tests were used for all orthogonal contrasts. Data from study 2, examining circulating levels of E2 in OVX females with E2 replacement were not statistically analyzed, as these data were meant to be descriptive.

3. Results

3.1 Latent Inhibition

Male rats exhibited LI regardless of HAL treatment (Fig 1A). A 2x3 ANOVA, with main factor of exposure (PE vs NPE) and HAL dose (vehicle, 0.05mg/kg, 0.1mg/kg), was used to analyze data from the male rats. This ANOVA revealed a significant main effect of exposure, $F(1, 52) = 19.41$, $p<0.001$. This main effect was due to significantly higher suppression ratios in the PE group, compared to the NPE group, indicating that LI occurred. Orthogonal contrasts demonstrated that the PE rats had significantly higher suppression ratios than the NPE rats in the vehicle group ($t(20) = 2.42$, $p=0.023$), the 0.05mg/kg HAL group ($t(16) = 3.13$, $p=0.004$), and the 0.1mg/kg HAL group ($t(16) = 3.77$, $p=0.002$). This shows that male rats developed LI regardless of the dose of HAL.

A 2x3x3 ANOVA, with main factors of exposure (PE vs NPE), E2 replacement (no E2, low E2, and high E2), and HAL dose (vehicle, 0.05mg/kg, 0.1mg/kg), was used to analyze data from the female rats. There was a main effect of exposure, $F(1, 173) = 27.37$, $p<0.001$, a main effect of HAL treatment, $F(2, 173) = 6.12$, $p=0.003$), and an interaction between exposure and HAL treatment, $F(4, 173) = 2.74$, $p=0.030$. Orthogonal contrasts demonstrated that OVX females receiving no E2 replacement in the PE group had significantly higher suppression ratios than NPE group in the vehicle condition ($t(25) = 2.32$, $p=0.029$), the 0.05mg/kg HAL condition ($t(22) = 2.45$, $p=0.023$), and the 0.1mg/kg condition ($t(16) = 2.92$, $p=0.010$). These OVX females showed a similar pattern of results to the male rats, exhibiting LI regardless of the dose of HAL.

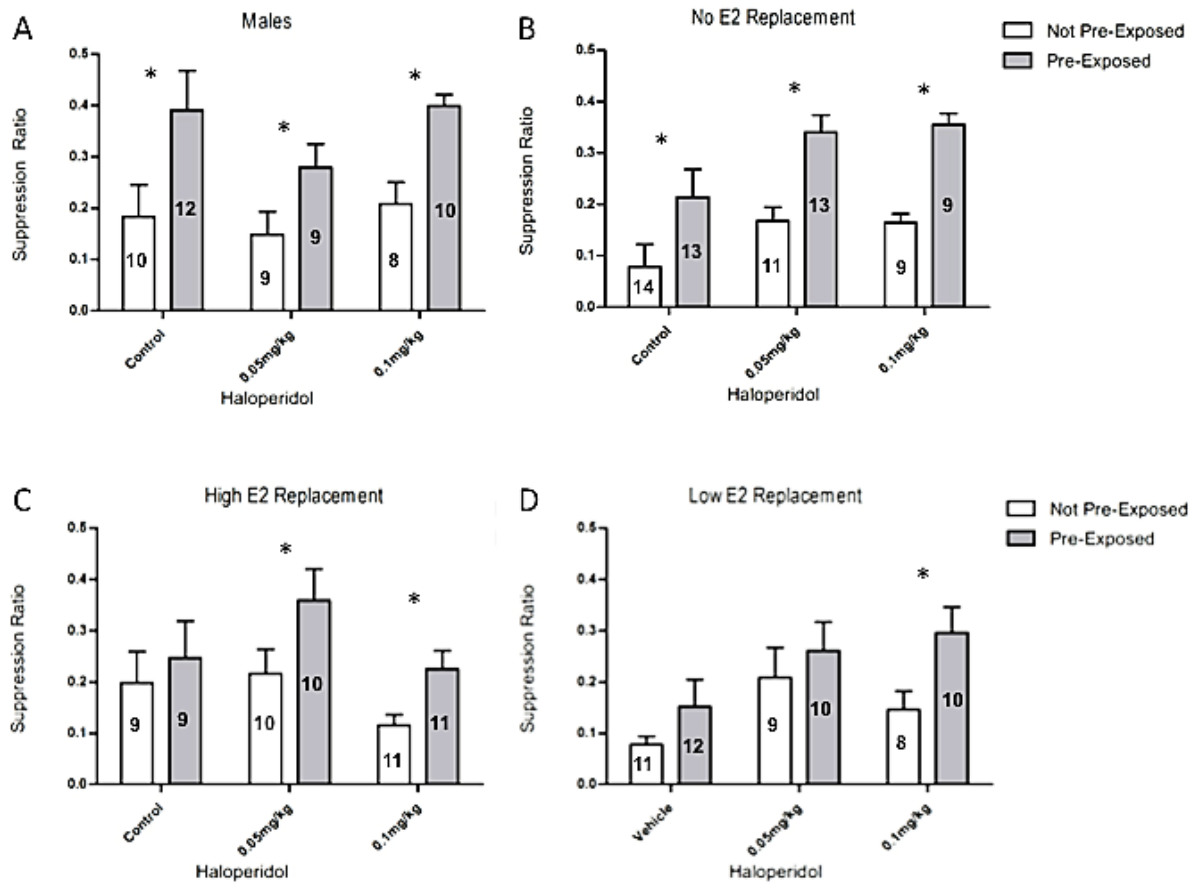

Figure 1. **A)** Suppression ratios for males; latent inhibition was observed in rats treated with 0.05 and 0.1 mg/kg haloperidol. **B)** Suppression ratios for OVX females receiving no E2 replacement; latent inhibition was observed in rats treated with vehicle, 0.05mg/kg, and 0.1 mg/kg haloperidol. **C)** Suppression ratios for ovariectomized (OVX) females receiving high estradiol (E2) replacement; latent inhibition was observed in rats treated with 0.05mg/kg and 0.1mg/kg haloperidol. **D)** Suppression ratios for OVX females receiving low E2 replacement: latent inhibition was observed in rats treated with the 0.1mg/kg haloperidol. * = $p<0.05$

Orthogonal contrasts demonstrated that OVX rats with either high or low E2 replacement did not develop LI when administered a vehicle injection. At the 0.05 mg/kg dose of HAL, rats in the high E2 group exhibited LI, that is, the PE rats exhibited significantly higher suppression ratios than the NPE rats ($t(10) = 2.35$, $p=0.03$). In contrast, rats receiving the low E2 replacement and the 0.05 mg/kg dose of HAL did not express LI insofar as there was no difference in suppression rations between PE and NPE groups. Finally, the 0.1mg/kg dose of HAL recovered LI in rats receiving both the high and low E2 replacement ($t(11) = 2.69$, $p=0.02$ and $t(10) =2.49$, $p=0.02$, respectively).

3.2 E2 Plasma Levels

The plasma concentrations of E2 following implantation of the silastic capsule were determined (Fig 2A). Data are expressed as mean ± standard error of the mean. Female rats had relatively low levels of E2 after ovariectomy, prior to implantation of the capsule (6.51±1.38pg/ml). There was an increase in the plasma concentration of E2 at week 1 (37.54±5.67pg.ml) and week 2 (38.25±11.72pg/ml) following capsule implantation. Plasma E2 levels slowly decreased at week 3 (29.41±16.58pg/ml) and week 4 (17.09±4.01pg/ml). These plasma concentrations are in the range of the average plasma level of E2 during diestrus in the rat (Overpeck et al, 1978).

A second experiment examined the plasma concentration of E2 in response to a silastic capsule implant paired with a 10ug/kg injection of E2 (Fig 2B). Rats had similar E2 plasma concentrations following capsule implantation as was observed in the previous experiment (28.66±5.91pg/ml), confirming the efficacy of the capsules. Four hours following the E2 injection the average plasma level of E2 increased markedly to 208.79±12.03pg/ml. The plasma concentration of E2 had decreased to 79.00±4.30pg/ml 8 hrs following the injection, and was maintained close to that level 12hrs following injection (83.26±24.08pg/ml). Sixteen hours after the injection plasma concentrations of E2 dropped to 51.30±11.68pg/ml, and 20hrs following the injection plasma levels were down to 39.18±8.97pg/ml (Fig 2B). The average plasma concentration across the 20hr period following E2 injection is in the range of plasma levels observed during the proestrus phase of the cycle in the rat (Overpeck et al, 1978).

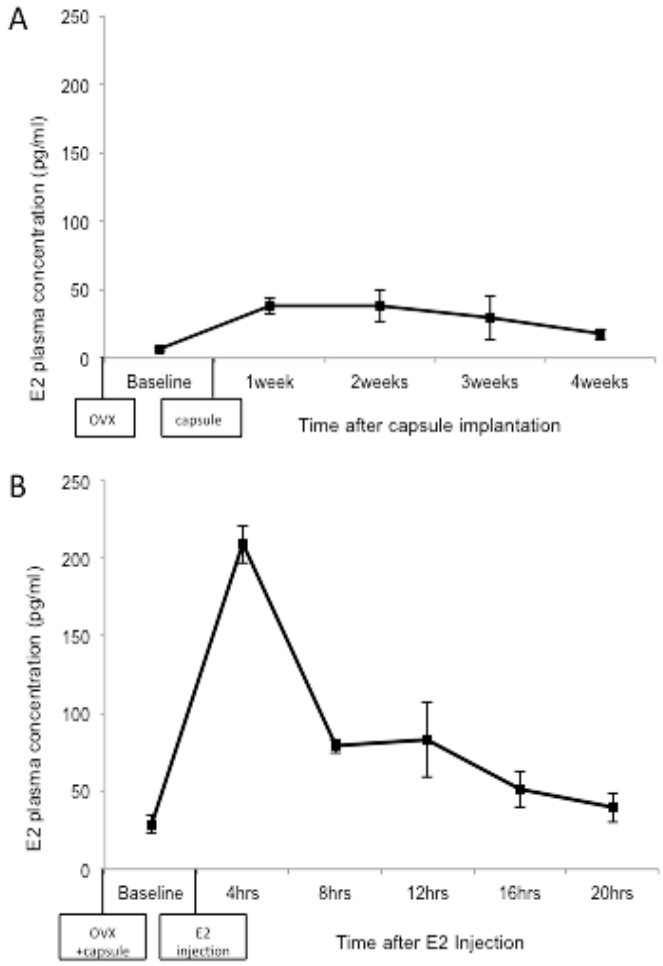

Figure 2. Estradiol (E2) plasma levels following: **A)** ovariectomy and implantation of a silastic capsule containing 5% 17-beta E2 and cholesterol. **B)** ovariectomy, implantation of a silastic capsule, and a 10ug/kg subcutaneous injection of E2, dissolved in sesame oil.

4. Discussion

These experiments demonstrate that physiologically relevant levels of E2 disrupt LI, and this disruption can be reversed by an acute injection of HAL administered prior to the conditioning session. Furthermore, the lower dose of HAL (0.05mg/kg) was sufficient to restore LI in the high E2 replacement rats, but not in those with low E2 replacement. This indicates that estrogens may facilitate the effects of HAL, as HAL is more effective in females with high levels of E2. Females are more responsive to antipsychotics than males (Seeman, 2004), and these results suggest that estrogens contribute to the differing responses to HAL in males and females in an LI paradigm. These experiments also show that the dose and method of E2 capsule replacement used here results in sustained low levels of E2 similar to that observed during diestrus for up to four weeks. Furthermore, the single injection of E2 shows a peak plasma concentration within four hours following injection, returning to baseline levels within twenty hours mimicking estrogen levels during proestrus (Overpeck et al 1978).

4.1 E2 and Latent Inhibition

The results presented here are in accordance with previous research from this lab indicating that estrogens disrupt LI in females under conditions where LI is observed in males and in OVX female rats (Nofrey et al, 2008; Quinlan et al, 2010). When OVX rats are administered E2 replacement alone, there is no difference between suppression ratios in the PE and NPE groups, indicating that LI did not occur (Fig 1). No difference was observed between PE and NPE females due to low suppression ratios in the PE group, suggesting that the E2 treated females are unable to ignore the tone that should have been rendered irrelevant through multiple non-reinforced presentations. In contrast both male rats and OVX female rats given no E2 replacement have significantly higher suppression ratios in the PE group compared to the NPE group, indicating that LI did occur. Taken together these findings demonstrate that estrogen has detrimental effects on selective attention, impairing the ability to disregard irrelevant stimuli in the environment.

In contrast to these findings there is a body of research indicating that ovariectomy induces deficits in LI while E2 facilitates LI (Arad and Weiner, 2009; 2010a; 2010b). The reason for these contradictory results is unclear, although methodological differences likely contributed. In the conditioned emotional response LI paradigm employed by others (Arad and Weiner, 2009; 2010a; 2010b) they use a 10 sec, 80dB tone during pre-exposure and conditioning compared to

the 1 sec 65dB tone used in our experiments. Therefore their stimuli is much more salient, since it is louder and longer, which could impact whether the stimuli is rendered irrelevant during the preexposure phase, and the association formed during the conditioning phase. Additionally, the high E2 replacement dose used here is much lower than the E2 dose used by other researchers in the field. In this study, efforts were made to use an E2 replacement regime that mimicked the estrous cycle of the rats; study 2 demonstrates that plasma levels of E2 following capsules implantation and capsule implantation combined with an acute injection E2 were within the range observed across the estrous cycle (Fig 2; Overpeck et al, 1978), so the results of this experiment should mimic the effects of endogenous estrogens. This difference in the E2 replacement doses may provide a partial explanation for the contradictory findings on the effects of E2 on selective attention.

4.2 Haloperidol and LI

It is well established that HAL can facilitate LI in male rats (Moser et al, 2000), and more recently research has demonstrated that this is also true for female rats (Arad and Weiner, 2009). The findings of the current experiments correspond to this, as the highest dose of HAL restores LI in females receiving both low and high E2 replacement. More interestingly these results indicate that E2 actually facilitates the effects of HAL on LI, as the lower dose of HAL was sufficient to restore LI in the high E2 replacement group, but not in the low E2 replacement group. In these experiments, and others (Arad and Weiner, 2008; Nofrey et al, 2008; Quinlan et al, 2010), elevated levels of circulating estrogens are detrimental to LI in female rats, but these findings indicate that E2 replacement also facilitates the effects of HAL treatment in facilitating LI. There is evidence that this might be the case in humans as well. Sex differences in LI are observed in humans, where males exhibit LI under conditions where females do not (Vol'f et al., 2001; Kaplan and Lubow, 2011), and the peak of estrogens in the menstrual cycle is correlated with distractibility and an inability to disengage from irrelevant stimuli (Beaudoin and Morrocco, 2003). Additionally, research in humans indicates that antipsychotic treatments are more effective in females, as schizophrenic women require significantly lower doses of antipsychotic drugs to alleviate their symptoms than men (Usall et al, 2003). Furthermore E2 administered in conjunction with antipsychotic treatment results in better treatment outcomes in schizophrenia (Akhondzadeh et al, 2003; Seeman, 2004).

This study did not examine the neurobiological underpinnings of the behaviours observed in the LI paradigm. However, previous research has implicated dopaminergic transmission in various nuclei of the mesocorticolimbic system in LI. In the PFC, lesions and local infusion of a dopamine antagonist enhance LI (Broersen et al, 1996; George et al, 2010). Lesions of the NAc shell abolish LI (Gal, Schiller and Weiner, 2005; Weiner et al., 1996), lesions to the NAc core result in abnormally persistent LI (Weiner et al., 1996), and lesions of both the NAc core and shell result in enhanced LI (Gal, Schiller and Weiner, 2005). Additionally, dopaminergic activity in the anterior STR is positively correlated with behaviour in an LI task (Jeanblanc et al, 2003). Interestingly, estrogens have been shown to alter dopamine transmission in these nuclei of the mesocorticolimbic system. Specifically, estrogens increase dopamine levels and D2 receptor density in the NAc (Le Saux et al, 2006; Thompson and Moss, 1994), increase dopamine activity and D2 receptor density in the STR (Becker and Rudick, 1999; Landry et al, 2002), and increase dendritic spine and dopamine 1 receptor density in the PFC (Lévesque and Di Paolo, 1989; Wallace et al, 2006). Estrogen-induced changes in dopamine transmission in the mesocorticolimbic system could be partially responsible for the detrimental effects of estrogens on behaviour in the LI paradigm. Additionally, HAL exerts its effects by antagonising D2Rs, and E2 replacement increases in D2R density in the STR and NAc (Landry et al, 2002; Le Saux et al, 2006). Therefore, E2 replacement would increase binding sites for HAL, providing one possible explanation for estrogens' facilitatory effects on this antipsychotic. Future research should investigate these hypotheses to determine whether estrogen-induced changes in dopaminergic transmission in the mesocorticolimbic system are responsible for the E2-induced reductions in selective attention, and increased response to HAL treatment, observed in this study.

4.3 Conclusions

E2 replacement administered to OVX females abolishes LI under conditions where it is observed in males, but despite these detrimental effects on LI, E2 facilitates HAL to enhance LI. These experiments indicate that estrogens are, in part, responsible for sex differences in LI and the response to HAL. Further research is needed to extend these findings from HAL to other antipsychotic treatments, and to elucidate the specific neurobiological mechanisms responsible for estrogens effects on LI and the response to antipsychotic treatments.

CHAPTER 3:

ESTRADIOL FACILITATES THE EFFECTS OF HALOPERIDOL TO REDUCE PERSEVERATION AND FACILITATE REVERSAL LEARNING IN AMPHETAMINE-SENSITIZED FEMALE RATS

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Preface:

The study in Chapter 3 attempts to extend the findings from the LI study to other cognitive processes that are affected in individuals with schizophrenia. Specifically, the experiments in Chapter 3 examine the effects of estrogens, alone and in conjunction with HAL, on perseveration and reversal learning. The methodology in these experiments was adjusted to increase ecological validity; HAL was administered chronically, via osmotic minipump, using a dose that was previously shown to achieve plasma levels that mimic those observed in humans administered HAL (Featherstone, Kapur, and Fletcher, 2007). Additionally, all rats in the perseveration and reversal learning experiments were amphetamine sensitized to mimic the reversal learning deficits and excessive perseveration observed in schizophrenia (Samaha, Seeman, Stewart, Rajabi, and Kapur, 2007).

Abstract:

There are sex differences associated with schizophrenia, where women have later onset of the disorder, less severe symptoms, and better response to antipsychotic medications. Estrogens are thought to play a role in these sex differences, and there is evidence that they protect against the positive symptoms of schizophrenia, but it remains unclear whether estrogens also protect against cognitive symptoms of schizophrenia. Amphetamine (AMPH) sensitization of locomotor activity is used as an animal model for some of the symptoms of schizophrenia, including cognitive deficits such as excessive perseveration and slower reversal learning. This experiment used OVX amphetamine sensitized female rats to investigate the effects of E2 and the antipsychotic HAL, administered alone and in combination, on perseveration and reversal learning. Perseveration and reversal learning were measured in operant chambers where rats were trained to press one of two levers to receive a sucrose pellet. Perseveration was assessed as the number of lever presses made during a one day extinction test, when levers pressing no longer resulted in delivery of sucrose, and reversal learning was assessed as the time to switch from pressing the formerly active lever to the newly activated lever. Results of these experiments demonstrated that E2 alone did not affect either perseveration or reversal learning, but indicate that E2 facilitates the effects of HAL to reduce perseveration and improve reversal learning. These results suggest that the optimal dose HAL differs based on serum levels of E2.

1. Introduction:

There are sex differences in the progression of schizophrenia and the response to antipsychotic drugs, with women exhibiting later onset of the disorder, and reduced symptom severity (Hafner, 2003; Seeman, 1982). Additionally, natural declines in plasma estrogen levels pre-menstruation (Endo, Daiguji, Asano, Yamashita, and Takahashi, 1978; Glick & Stewart, 1980), post-partum (Kendell, Chalmers, & Platz, 1987; McNeil, 1987), and following menopause (McNeil, 1987), are associated with increased vulnerability to psychosis. In addition to protecting against psychosis, estrogens are also linked to better treatment outcomes (Chua, de Izquierdo, Kulkarni, and Mortimer, 2005; Kulkarni, Gavrilidis, Worsley, and Hayes, 2012; Seeman, 1982); women require lower doses of antipsychotics to treat both acute (Chouinard & Annable, 1982; Chouinard & Turnier, 1986) and chronic, schizophrenia (Seeman, 1982; Seeman and Lang, 1990). Recent clinical experiments demonstrate that women administered E2 in conjunction with antipsychotic medication exhibited less positive, negative and general psychopathology symptoms compared to controls that received antipsychotic treatment alone (Akhondzadeh et al., 2003; Kulkarni et al., 2014). These experiments demonstrate the potential for estrogen in the improvement of the positive and negative symptoms of schizophrenia, but it remains unclear whether estrogens affect the cognitive symptoms of schizophrenia.

These experiments focused on two related cognitive symptoms of schizophrenia: excessive perseveration and deficits in reversal learning. Perseveration is a cognitive process defined by the repetition of a previously reinforced behavior, despite the fact that reinforcement is no longer provided (Crider, 1997; Holahan, Madularu, McConnell, Walsh, & DeRosa, 2011), and reversal learning is defined as the cognitive capacity to discontinue a behaviour that is no longer relevant in a particular context, and adopt a novel behaviour that reflects the contextual change (Pantelis et al., 1999). Patients with schizophrenia are capable of acquiring the initial rule but perform poorly once the rule changes; they perseverate (Waford & Lewine, 2010), persisting in the previously effective behavior, and demonstrate longer latencies to modify their behaviour to reflect a new rule (Pantelis et al., 1999; Reed, Harrow, Herbener, & Martin, 2002; Waltz & Gold, 2007). Typical antipsychotic medications, including HAL, effectively treat the positive symptoms of schizophrenia, but are frequently ineffective in alleviating the cognitive symptoms associated with this disorder (Bowie & Harvey, 2006). The cognitive symptoms of schizophrenia

are the best predictor of functional outcome (Bowie & Harvey, 2006), so there is an impetus to discover treatments that improve these symptoms.

Animal models have been developed for some of the symptoms of schizophrenia (Jones, Watson, & Fone, 2011) to allow for controlled experiments examining the efficacy of antipsychotic drugs. AMPH sensitization of locomotor activity is an animal model that induces some neurobiological and behavioural changes associated with schizophrenia (Featherstone, Kapur, & Fletcher, 2007). The dopamine hypothesis of schizophrenia states that this disorder is caused, in part, by elevated dopamine in the dorsal and ventral striatum, and reduced dopamine transmission in prefrontal regions (Abi-Dargham et al., 1998; Howes & Kapur, 2009). In humans, AMPH administration increases dopamine availability in the dorsal and ventral striatum (Boileau et al., 2006) and repeated AMPH use can cause psychosis (Bell, 1965). In rats, repeated AMPH administration also results in increased dopamine transmission in the NAc and STR, even after extended periods of abstinence from AMPH, a phenomenon known as neurobiological sensitization (Fiorino & Phillips, 1999; Paulson & Robinson, 1995). Together these findings suggest that repeated AMPH administration induces a neurobiological state and similar to that observed in schizophrenia. Interestingly, repeated AMPH administration to rodents also leads to increased locomotor activity in response to the same dose of drug, which is referred to as locomotor sensitization (Featherstone et al., 2007; Paulson & Robinson, 1995). Research has demonstrated that locomotor sensitization develops in parallel with neurobiological sensitization (Pierce & Kalivas, 1995), so it can be used as a behavioural marker for neurobiological sensitization.

There is behavioural evidence that AMPH sensitization of locomotor activity models some of the cognitive symptoms of schizophrenia. AMPH sensitization of locomotor activity induces deficits in LI in male rats, (Murphy, Fend, Russig, & Feldon, 2001; Russig, Murphy, & Feldon, 2002; Tenn, Kapur, & Fletcher, 2005) which are ameliorated by the antipsychotics HAL and clozapine (Russig et al., 2002). More pertinent to this study, an acute dose of AMPH increases perseveration in a Y-maze task in male rats (Hahn, Zacharko, & Anisman, 1986; Oades, Taghzouti, Simon, & Le Moal, 1985), which is ameliorated by HAL treatment (Oades et al., 1985). Similarly, acute AMPH administration induces deficits in reversal learning which are reversed by acute HAL treatment (Idris, Repeto, Neill, & Large, 2005). Repeated AMPH treatments also induces deficits in reversal learning in rats (Featherstone, Rizos, Kapur, &

Fletcher, 2008; Fletcher, Tenn, Rizos, Lovic, & Kapur, 2005) and Marmosets (Ridley, Haystead, & Baker, 1981), which are ameliorated by an acute administration of HAL (Ridley et al., 1981). These findings demonstrate that AMPH induces deficits in selective attention, reversal learning, and excessive perseveration, similar to those observed in schizophrenia, which are alleviated by administration of antipsychotic drugs. This suggests that sensitization of locomotor activity is a good model for some of the cognitive symptoms associated with schizophrenia.

Despite clinical research indicating that estrogens improve the efficacy of antipsychotic medication, little preclinical research has examined the possible interaction between estrogens and antipsychotic drugs. Previous research with OVX females suggests that administration of combined E2-HAL treatment improves selective attention significantly more than HAL alone (Almey, Hafez, Hantson, & Brake, 2013; Arad & Weiner, 2009), paralleling clinical research findings (Akhondzadeh et al., 2003; Kulkarni et al., 2014). However there is little previous research examining the effects of estrogen and HAL on perseveration or reversal learning. Perseveration can be assessed through extinction tasks, and it was shown that E2 administered to OVX rats facilitates extinction of lever pressing for cocaine, suggesting that E2 decreases perseveration (Twining, Tuscher, Doncheck, Frick, & Mueller, 2013). However, other experiments indicate that estrogens do not affect extinction of lever pressing for cocaine (Larson & Carroll, 2007). In terms of reversal learning, a physiologically relevant dose of E2 has no effect on this behaviour, while a supraphysiological dose of E2 administered alone or with HAL has detrimental effects on reversal learning (Arad & Weiner, 2012). Results from these experiments are contradictory and difficult to interpret based on methodological differences.

The experiments in this study attempt to clarify the effects of E2, administered alone and in conjunction with HAL, on perseveration and reversal learning in amphetamine sensitized rats. Both perseveration and reversal learning were assessed in operant boxes equipped with two levers, and rats were trained to press one of these levers to receive a sucrose pellet. To test perseveration neither lever delivered a pellet, and the extinction of lever pressing was used as a measure of perseveration. To assess reversal learning the lever that delivered sucrose pellets was switched, and the time it took for rats to adjust their behavior to match this new rule was used as a measure of reversal learning. It was predicted that E2 would have a detrimental effect on reversal learning and perseveration, but would facilitate the effects of HAL to decrease perseveration and improve reversal learning.

2. Methods

2.1 Subjects

The subjects consisted of 121 female Sprague-Dawley rats (for group sample sizes see Figures; Charles River Laboratories, St. Constant, QC), weighing 220-240g upon arrival. Rats were pair housed in standard clear shoebox cages in a colony room maintained on 12hr reverse light cycle at constant temperature (21ºC) and humidity (60%). One week before behavioral training, rats were housed individually and food restricted to 90% of their free feeding weight. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care, and approved by Animal Research Ethics Committee of Concordia University.

2.2 Surgery and Estradiol Replacement

Ovariectomy and capsule implantation. All rats were ovariectomized to control for naturally cycling estrogens. Ovaries were removed via lumbar incision under isofluorane gas anesthesia (4% induction, 2% maintenance). Rats were administered 0.1 mL of the analgesic Anafen (10mg/mL) by subcutaneous (SC) injection, and 0.1 mL of the antibiotic penicillin (30,000 IU/mL) by intramuscular injection. Rats were assigned to one of three groups: no E2 replacement, low E2 replacement, or high E2 replacement. During the ovariectomy rats in the low and high E2 replacement groups were implanted with a silastic capsule containing E2, described below.

Hormone Treatment. E2 replacement was administered via silastic capsule containing 5% 17-β E2 in cholesterol (Sigma-Aldrich, St.Louis, MO). These capsules produce a plasma concentration of ~20-25 pg/ml E2 (Almey et al., 2013), which corresponds to plasma levels of E2 observed during the diestrus phase of the estrous cycle (Butcher, Collins, & Fugo, 1974). In addition to capsules, rats in the high E2 replacement group received injections of E2 in sesame oil every four days (10 μg/kg, SC), starting at the beginning of the final training phase (see Procedures). The combination of the E2 capsule and this SC injection of E2 achieved an average plasma level of ~90pg/ml across 12 hours (Almey et al., 2013), similar to plasma levels of E2 during the proestrus phase of the cycle (Butcher et al., 1974). Rats in the no E2 and low E2 groups received sesame oil injections.

2.3 Drug treatments

Amphetamine. D-amphetamine sulphate (Sigma-Aldrich, St. Louis, MO) was repeatedly administered via intraperitoneal injection (IP) to induce locomotor sensitization. For the

induction phase of sensitization all rats were administered 1mg/kg AMPH daily for four consecutive days; locomotor activity was assessed for 1hr following AMPH administration. After induction all rats underwent a 7 day period when they received no AMPH, and then they were given an AMPH challenge (0.5mg/kg IP) and locomotor activity was assessed. Locomotor, and therefore neurobiological sensitization, was considered to have developed if rats exhibited comparable or higher levels of locomotor sensitization in response to the challenge dose of AMPH (0.5mg/kg) as they did to the initial dose of AMPH in induction (1mg/kg). This protocol has previously been shown to induce AMPH sensitization of locomotor activity (Madularu, Shams, & Brake, 2014).

Haloperidol treatment. Rats in each hormone condition were randomly divided into two drug treatment groups, one of which was implanted with osmotic minipumps (Alzet, model 2002; Durect, Cupertino, CA, USA) containing HAL, and a control group that were implanted with minipumps containing saline. For the perseveration and a first reversal learning experiment minipumps infused 0.25mg of HAL per day for 14 days. A second reversal learning experiment implanted minipumps that released 0.13mg HAL per day. Delivery 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 in humans (Samaha et al., 2008; Samaha, Seeman, Stewart, Rajabi, & Kapur, 2007). For minipump insertion rats were anesthetized with isoflurane, as described in the ovariectomy section, and minipumps were implanted subcutaneously through a small dorsal incision.

2.4 Materials

Operant conditioning chambers. Behavioral training and testing were conducted in operant chambers (25 cm x 30 cm x 30 cm; Coulborne Instruments, Allentown, PA) enclosed in sound-attenuating isolating units. Each chamber contained two levers located 5 cm above the grid floor. The levers were positioned symmetrically on each side of a food magazine, where sucrose pellets were delivered. Pressing the active lever resulted in the inactivation of the house light and the delivery of a 45 mg sucrose pellet (Bio-Serv). Pressing the inactive lever had no effect. Operant chambers were controlled by Graphic State software that recorded lever presses and food magazine entries for analysis (Coulborne Instruments, Allentown, PA).

Locomotor Activity Boxes. Locomotor activity during the AMPH sensitization procedure was assessed using locomotor activity boxes (42 cm x 25 cm x 17 cm). Locomotor activity was

assessed via two pairs of photocells at 14cm increments, located 2.5cm above the grid floor. Interruptions of the photobeams were recorded by a computer running custom designed software activity (Steve Cabilio, 1999), and were used as the measure of locomotor activity.

2.5 Procedure

For all three experiments rats were food restricted to 90% of their free feeding weight. Behavioral training took place during the dark phase of the diurnal cycle. Once rats reached 90% of their bodyweight shaping procedures began, which involved a daily 1hr session in operant chambers to train rats to press a lever for a chocolate flavoured sucrose pellet at a fixed ratio 1. For the perseveration experiment rats were placed in the operant chambers for 1hr shaping sessions, daily, where pressing either of the two levers (counterbalanced across rats) resulted in the delivery of reinforcement. Shaping continued until all rats were consistently pressing the lever (>100 presses per session) for at least 2 days; this took ~10 days of training. For the reversal learning experiments rats were placed in the operant chambers for 1hr training sessions, daily, where pressing both of the levers resulted in the delivery of a reinforcement. Once rats were consistently pressing the lever (>100 presses per session) the protocol was changed so that pressing only one of the levers resulted in delivery of reinforcement; the lever that delivered the reinforcement was alternated daily to ensure that rats did not develop a strong preference for either lever. Shaping continued until rats would press either lever >100 times per training session (~14 days).

Following shaping all rats were OVX as described above; rats in the low and high E2 groups were implanted with E2 capsules at this time. Rats were given one week to recover from surgery with ad libitum food. Following this the AMPH sensitization protocol began. For induction of locomotor sensitization all animals were treated with 1mg/kg AMPH, IP, daily for four consecutive days. Following a seven day withdrawal period, rats received a 0.5mg/kg challenge dose of AMPH and locomotor activity was assessed to determine if locomotor sensitization developed.

Immediately following the AMPH challenge, all rats were implanted with an osmotic minipump containing either saline or HAL. Rats were given one day to recover from minipump implantation, and then the final training began. During training one of the two levers was active, resulting in delivery of a sucrose reinforcement, while the other lever was inactive. In the perseverance experiment the active lever was the same as during shaping. In the reversal learning

experiments the active lever was counterbalanced across rats. Rats were trained for 1hr daily in the operant chambers for 10 consecutive days during which the active lever remained the same. It was during this final training period that E2 injections for high E2 replacement group were administered. Rats received E2 injections, SC, at ~4pm on day 2, 6, and 10 of training. Injections were given every fourth day to mimic the rat estrous cycle; E2 injections were administered the afternoon of day 10, ~16hrs before testing occurred, allowing time for the long-term effects of E2 to occur before the behavioural test.

On the 11th day testing for perseveration or reversal learning began. Thirty minutes before testing all rats were administered a 0.5mg/kg injection of AMPH (IP) before they were placed in the operant chambers. For the perseveration experiment both levers were inactive; regardless of any lever pressing no reinforcement was delivered. The measure of perseveration was the number of lever presses on the formerly active lever during the 1hr testing session. For the reversal learning experiment the formerly active lever was now inactive, and the inactive lever was made active. During testing, rats were expected to alter their pressing behavior in response to the change in reward contingency, such that they were expected to stop pressing the formerly active lever and switch to press on the previously inactive lever. Reversal testing lasted for two 1hr testing sessions on consecutive days (Day 11 and 12).

2.6 Statistical analysis.

In order to confirm that sensitization of locomotor activity occurred in response to AMPH treatment, locomotor activity in response to the 0.5 mg/kg AMPH challenge was required to be equal to or greater than locomotor activity in response to the initial 1 mg/kg dose of AMPH. A two-tailed dependent sample t-test was employed to compare differences in locomotor activity. Sensitization of locomtor activity was considered to have developed if rats exhibited similar or significantly higher locomotor activity to the 0.5mg/kg AMPH challenge than they did to the initial 1mg/kg dose of AMPH during induction.

For perseveration, the dependent variable was a ratio calculated between the number of active lever presses during the extinction challenge (A) and the average number of active lever presses over the last two days of training (B; A/(A+B)); this ratio depicted the number of lever presses on the formerly active lever, while controlling for individual differences in lever pressing behaviour. For reversal learning, the dependent variable was measured using a ratio of active lever presses (A) to total lever presses (B; A/(A+B)). Reversal learning was measured as the

latency to modify pressing behavior in response to the newly implemented reward contingency. The criterion for having achieved successful reversal learning was defined as achieving 90% of the lever press ratio attained on the last day of training for three consecutive 5min time bins. This criterion was used to ensure rats had consistently reversed their lever pressing behaviour.

In order to assess the effects of HAL on perseveration and reversal learning planned orthogonal contrasts were conducted with independent samples t-tests comparing HAL and saline treated rats in each hormone condition (no E2, low E2, and high E2). Cohen's *d* effect sizes with confidence intervals were calculated for each planned comparison. Three 2 x 3 ANOVAs were used to compare the drug and hormone treatment conditions; one ANOVA compared perseveration ratios between treatment conditions, and two ANOVAs compared time to achieve reversal criterion (min): one for the 0.25mg HAL data and one for the 0.13mg HAL. Tukey's post-hoc analyses were used to explore any significant main effects or interaction effects.

3. Results

The AMPH treatment regime induced locomotor sensitization in all hormone conditions (Fig 1). In the perseveration experiment there was no significant difference in locomotor activity to the AMPH challenge (0.5mg/kg) and the initial induction dose of AMPH (1mg/kg) in the no E2 and high E2 conditions; in the low E2 condition there was significantly higher locomotor activity to the challenge dose of AMPH than the induction dose of AMPH, $t(15) = -3.23$, $p < 0.01$ (Fig 1A). In the reversal learning experiment using 0.25mg HAL the no, low, and high E2 groups exhibited greater locomotor activity to the challenge dose of AMPH than to the induction dose, $t(12) = -3.86$, $p < 0.01$, $t(11) = -2.42$, $p < 0.05$, and $t(14) = -2.95$, $p < 0.05$, respectively (Fig 1B). Finally, in the reversal learning experiments using the 0.13mg dose of HAL, the no and low E2 groups had no significant difference in locomotor activity between induction and challenge doses of AMPH, while the high E2 group exhibited significantly more locomotor activity to the challenge dose than the induction dose of AMPH, $t(17) = -3.58$, $p < 0.01$ (Fig 1C). These findings demonstrate that the AMPH sensitization regime induced locomotor sensitization in all groups, indicating that neurobiological sensitization also occurred.

In the perseveration experiment the 2x3 ANOVA revealed a main effect of HAL, $F(1,39) = 14.02$, $p = 0.001$, with HAL treated rats ($M = 0.44$) exhibiting significantly less lever pressing

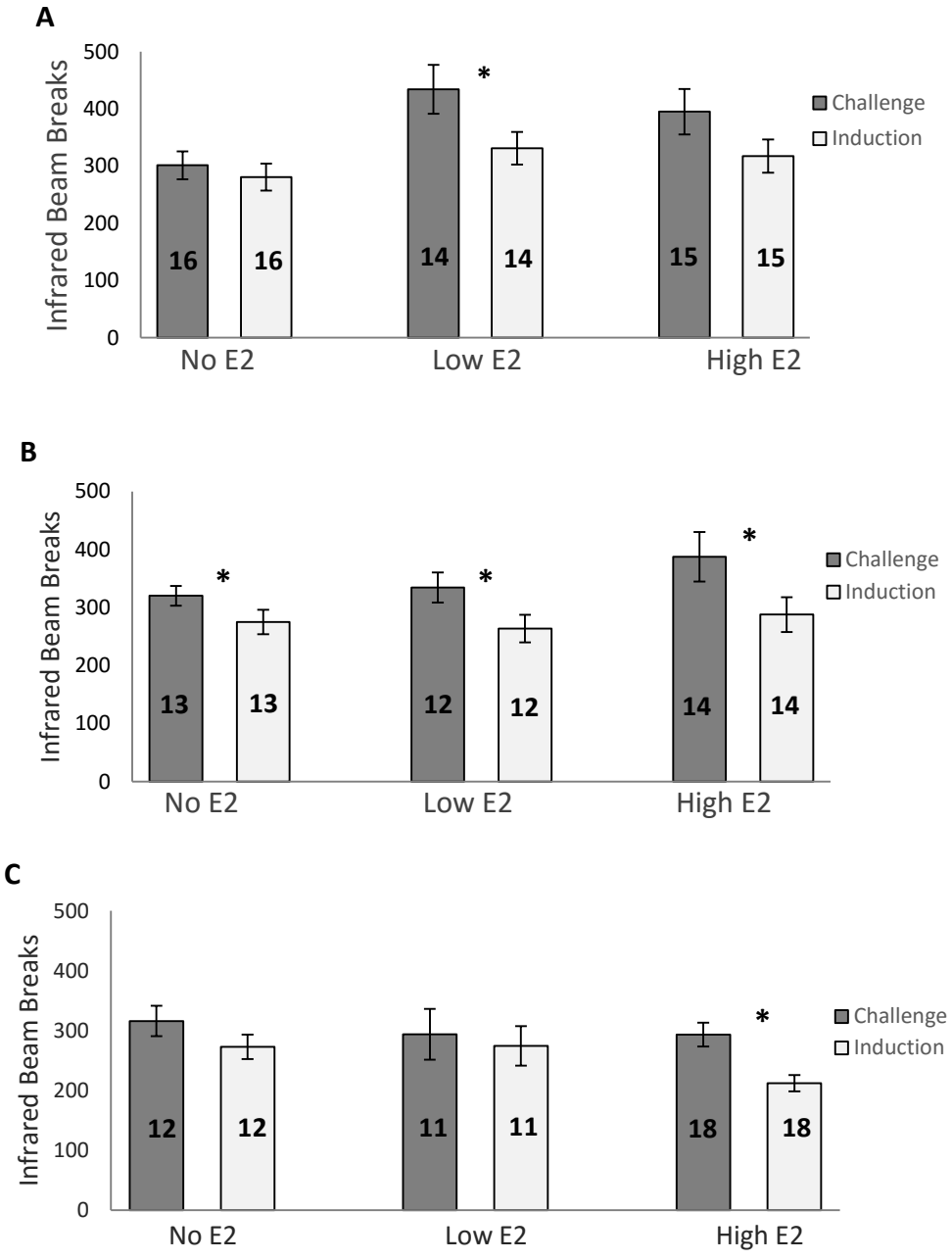

Figure 1. Locomotor sensitization data from no E2, low E2 and high E2 rats in: **A)** the perseveration experiment, **B)** the reversal learning experiment with 0.25mg HAL, **C)** the reversal learning experiment with 0.13mg HAL. Error bars depict the standard error of the mean, * = $p <$ 0.05, numbers on bars are the *n* for each group.

than SAL treated rats (M = 0.56; Fig 2)). There was no main effect of hormone treatment group, and no interaction between drug and hormone treatments. Orthogonal planned comparisons demonstrated that there was no significant difference between HAL and SAL treated rats in the no E2 condition, d = 0.43, CI ± 1.48. However, HAL treated rats in the low E2 conditions exhibited significantly lower lever pressing than SAL treated rats, $t(12) = 3.71$, $p < 0.01$, d = 1.98, CI ± 1.46. In contrast, there was not a significant difference between SAL and HAL treated rats in the high E2 condition, although there was a trend towards HAL treated rats perseverating less than SAL treated rats, $t(13) = 2.11$, $p = 0.054$, $d = 1.09$, CI ± 1.21. Although the chronic 0.25mg dose of HAL did not decrease perseverative responding in OVX females with no E2 or high E2 replacement, HAL administered in conjunction with a low chronic E2 replacement regime significantly reduced perseverative responding in AMPH sensitized female rats (see Fig 2).

In the first reversal learning experiment, using a 0.25mg dose of HAL, a 2x3 ANOVA revealed a significant main effect of drug $F(1,32)=5.52$, $p<0.05$, with HAL treated rats (M=53.06min) exhibiting significantly faster reversal learning than SAL treated rats (M=73.25min). There was also a significant interaction between drug and hormone treatments, $F(2,32)=6.31$, $p<0.01$. Post hoc analyses demonstrated that rats administered no E2 and HAL (M=40.83min) and low E2 and HAL (M=35.00min) had significantly faster reversal learning than rats administered high E2 and HAL (M=83.33min), $p<0.01$. Post hoc analyses indicated no significant differences between SAL treated rats in the different E2 replacement conditions, suggesting that E2 had no effect on reversal learning. Orthogonal planned comparisons were used to compare HAL and SAL treated rats in each hormone condition. In the no E2 and high E2 conditions there was no significant difference in reversal learning between HAL and SAL treated rats, d = 0.66, CI ± 1.10 and d = 0.74, CI ± 1.27, respectively. In contrast, in the low E2 condition rats administered HAL demonstrated significantly faster reversal learning than SAL treated rats $t(11) = 3.72$, $p < 0.01$, $d = 2.15$, CI ± 1.67. These results demonstrate that, in general, HAL treated rats have faster reversal learning. However, this effect is driven by rats in the low E2 condition, as there are no significant differences between HAL and SAL treated rats in the no and high E2 conditions (see Fig 3A).

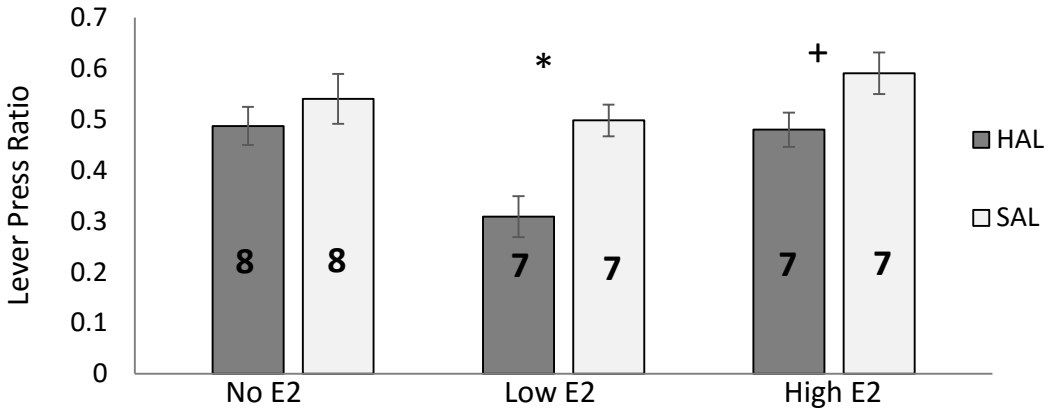

Figure 2. Lever press ratio for haloperidol (HAL) or saline (SAL) treated ovariectomized rats that received no, low and high E2 replacement. Error bars depict standard error of the mean, * = significant difference ($p < 0.05$), + = trend towards difference ($p < 0.1$), numbers on bars are the *n* for each group.

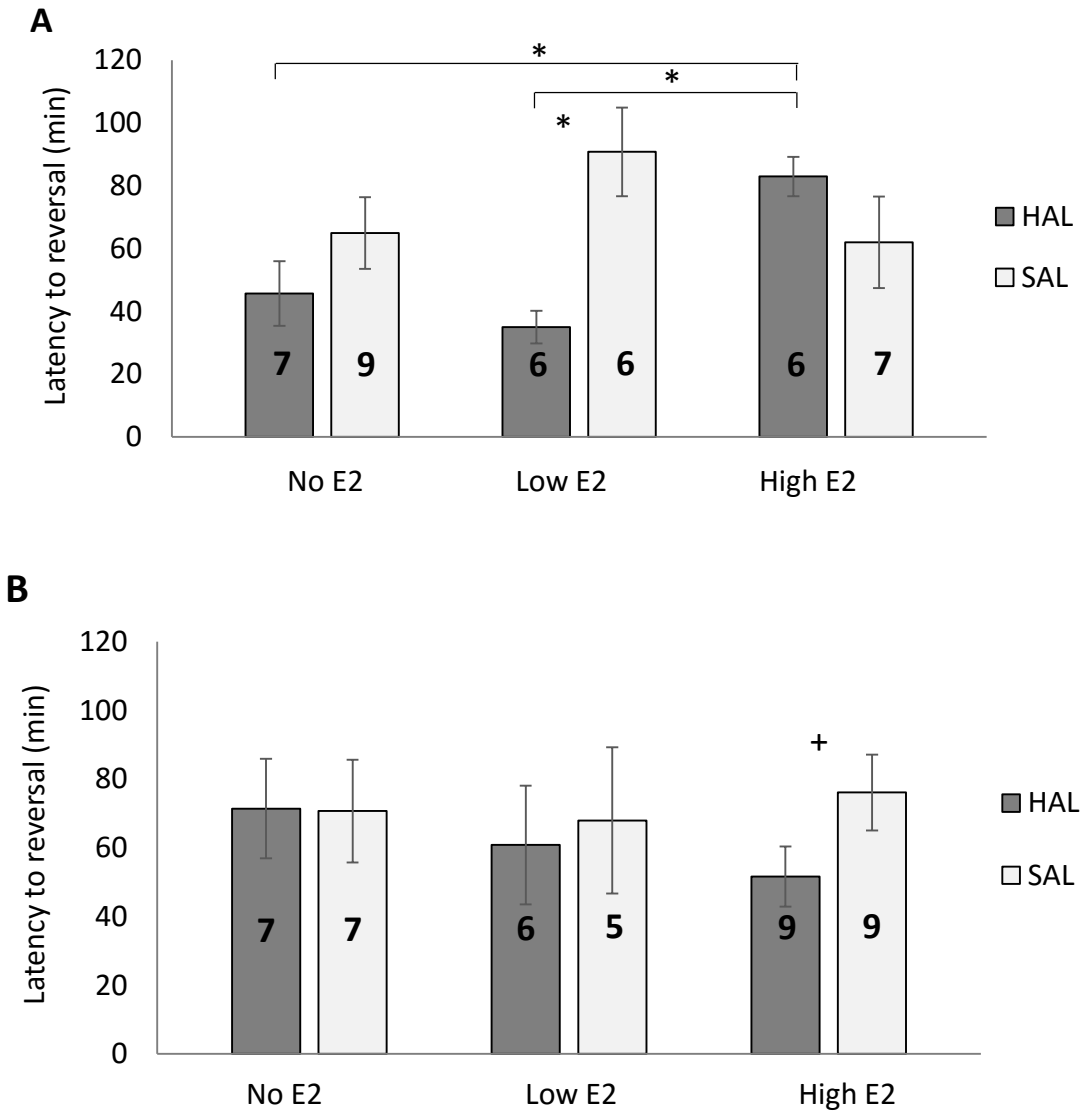

Figure 3. Time to achieve reversal criterion for no, low, and high E2 replacement groups that received **A)** the 0.25mg dose of haloperidol (HAL) or saline (SAL) and **B)** the 0.13mg dose of HAL or SAL. Error bars depict standard error of the mean, * = significant difference ($p < 0.05$), + = trend towards difference ($p < 0.1$).

In the second reversal learning experiment, which used a 0.13mg does of HAL, a 2x3 ANOVA no significant main effects and no significant interaction between the drug and hormone treatments. The orthogonal contrasts on each hormone condition demonstrated that there were no significant differences between HAL and SAL treated rats in the no and low E2 conditions, d = -0.02, CI ± 1.17 and d = 0.17, CI ± 1.37, respectively. However, in the high E2 condition there was a trend towards HAL treated rats exhibiting significantly faster reversal learning than SAL treated rats, $t(16) = 1.74$, $p = 0.10$, $d = 0.82$, CI ± 1.04. The results of this experiment indicate that a chronic 0.13mg dose of HAL is not sufficient to affect reversal learning behavior in no and low E2 treated female rats, but may reduce the latency to reversal behaviours in rats administered high E2 replacement (see Fig 3B).

4. Discussion

The results of these three experiments indicate that E2 alone has no effect on perseveration or reversal learning, while HAL generally reduces perseveration and improves reversal learning, although this effect is modified by the hormone replacement regime. These results also suggest that E2 facilitates the effects of HAL to decrease perseveration and improve reversal learning in AMPH sensitized female rats. In the perseveration and reversal learning experiments with the 0.25mg dose of HAL, this dose was not sufficient to affect perseveration or reversal learning in AMPH sensitized OVX rats that were not administered E2 replacement. However, in AMPH sensitized rats administered low E2 replacement, the 0.25mg dose of HAL decreased perseveration and the latency to reverse lever pressing behavior. In sensitized rats administered high E2 replacement and 0.25mg HAL, results were more difficult to interpret since this dose did not significantly improve perseveration or reversal learning. Previous research indicates that there is an optimal range of D2 receptor activation for successful reversal learning, so it is postulated that the combined 0.25mg HAL and high E2 replacement doses decreased dopamine transmission excessively. This hypothesis is supported by the results of the reversal learning experiment using the 0.13mg dose of HAL, discussed in detail below.

4.1 Methodological Considerations

The method of HAL administration, via 14 day release osmotic minipumps, imposed some restrictions on the behavioural protocols used in these experiments. Specifically, the length

of training and testing was determined by the longevity of the minipumps, since the animals had to be tested while they were receiving a full daily dose of HAL. Although the minipumps released the same volume (12µ) daily for 14 days, we wanted to conclude testing 12 days following minipump insertion to ensure that all rats were receiving a consistent dose of HAL. Additionally, rats were allowed one day to recover following minipump implantation before the daily final training and testing sessions began. Therefore, minipumps were inserted the afternoon of day 0, rats recovered day one, were trained for nine days (days 2-10), and testing started day 11. In a pilot study we observed that this 9 day period was not sufficient for all rats acquire consistent lever pressing behaviour. Because we could not extend this training period due to the time restrictions imposed by the minipumps we decided to shape lever pressing behaviour prior to beginning the experiment. This meant that all rats had acquired lever pressing, or had been excluded from the study, prior to ovariectomy surgeries and AMPH sensitization of locomotor activity.

It should be noted that in the shaping phase of the reversal learning experiment, once lever pressing behaviour was acquired the active lever was switched daily meaning that all rats had learned that either lever could provide reinforcement. This was done to ensure that no strong spatial bias was developed and reversal of lever pressing occurred relatively rapidly, so the majority of rats achieved reversal criteria during the two days of testing. Despite the fact that rats had obtained reinforcement for pressing the "inactive lever" during shaping, the behaviour observed during testing qualifies as reversal learning, since the rats had consistently performed one behavioural response for nine days before they were required to reverse that behavioural response during testing. This is supported by other studies that reverse behaviour multiple times during an experiment (Abdul-Monim, Reynolds, and Neill; Boulougouris, Dalley, and Robbins, 2007; Widholm, Clarkson, Strupp, Crofton, Seegal, and Schantz, 2001).

4.2 Locomotor Sensitization

In all experiments the rats exhibited locomotor sensitization, which indicates that neurobiological sensitization occurred in all groups. The effects of AMPH sensitization of locomotor activity on perseveration and reversal learning were not assessed in these experiments, but extensive previous research suggests that both acute and repeated AMPH administration increases perseveration and induces deficits in reversal learning (Featherstone et al., 2008;

Fletcher et al., 2005; Hahn et al., 1986; Idris et al., 2005; Oades et al., 1985; Ridley et al., 1981). Thus, it is assumed that the AMPH sensitization regimen used in these experiments induced similar deficits.

4.3 The effects of HAL and E2 on Perseveration and Reversal Learning

In general, the 0.25mg dose of HAL reduced AMPH-induced increases in perseveration and deficits in reversal learning, evidenced by the main effect of the 0.25mg dose of HAL observed in both the perseveration and reversal learning experiments. This parallels previous findings, which demonstrate that acute administration of HAL recovers AMPH induced increases in perseveration (Oades et al., 1985) and deficits in reversal learning (Idris et al., 2005; Ridley et al., 1981). To our knowledge, this is the first time a chronic HAL treatment regime, which mimics steady state plasma levels of HAL achieved with human antipsychotic treatment regimens (Samaha et al., 2008; Samaha et al., 2007), has been shown to decrease perseveration and improve reversal learning in an animal model of schizophrenia.

In contrast, there were no effects of E2 replacement on perseveration or reversal learning in AMPH sensitized female rats. There was no main effect of E2 replacement in the analyses on the perseveration or reversal learning data. In the reversal learning experiment with 0.25mg HAL, post-hoc analyses found no significant differences between SAL rats in the no, low, and high E2 replacement conditions. This corresponds to some previous research that showed no effect of E2 on perseveration, as measured by lever pressing for cocaine (Larson & Carroll, 2007). Our findings differ from other experiments which found that E2 administration decreased perseveration of lever pressing for cocaine (Twining et al., 2013) and impaired reversal learning (Arad & Weiner, 2012). There are numerous methodological differences between these experiments which could explain these discrepancies. For example, differences in the reinforcement provided in the task (cocaine vs. sucrose), differences in the behavior required (place preference vs. self-administration), and/or differences in E2 replacement doses (150µg/kg vs. 10µg/kg), or the fact that all rats in this study were amphetamine sensitized, could explain the incongruities between previous findings and the findings of the current study. These results indicate that physiological E2 replacement regimens have no effect on perseveration or reversal learning in AMPH sensitized female rats.

In the perseveration experiment and the reversal learning experiment that used the 0.25mg dose of HAL the low E2 replacement facilitated the effects of HAL in AMPH sensitized

female rats. In OVX female rats receiving no E2 replacement 0.25mg HAL did not affect perseveration or reversal learning, as HAL and SAL treated rats had comparable behavioural results. However, in the low E2 replacement group HAL treated rats exhibited significantly less perseverative responding and more rapid reversal learning than SAL controls, indicating that the low E2 replacement regime facilitated HAL. In contrast, there was no effect of this dose of HAL in rats administered high E2 replacement on perseveration or reversal learning. Interestingly, in the perseveration experiment there was a trend towards rats receiving the high E2 and 0.25mg dose of HAL perseverating less than SAL controls. In contrast, in the reversal learning experiment, rats administered this dose of HAL with the high E2 replacement actually had slower reversal learning than SAL controls, although this did not reach significance. It is unclear why the combined high E2 replacement and 0.25mg HAL treatment is more detrimental to reversal learning than perseveration; one possibility is that perseveration is a more simplistic cognitive process, so is less sensitive to E2-induced fluctuations in dopamine transmission than reversal learning, but further research is needed to address this. Initially, the results obtained when rats were administered the 0.25mg dose of HAL and high E2 replacement appear difficult to reconcile, but may be explained by evidence suggesting an inverted U-shaped curve of D2 receptor activation on perseveration and reversal learning.

4.4 An optimal level of D2 receptor activation for cognition

HAL ameliorates deficits in reversal learning induced by AMPH (Idris et al., 2005; Ridley et al., 1981), but chronic administration of HAL to rats that are not AMPH sensitized, and thus do not have excessive dopamine transmission, induces deficits in reversal learning (De Steno & Schmauss, 2009). Conversely, the administration of a D2 agonist to rats can also result in impairments in reversal learning (Boulougouris, Castane, & Robbins, 2009). Additionally, both D2 agonist and antagonist drugs infused into the forebrain of pigeons cause increases in perseverative errors in a reversal learning task (Herold, 2010). Together these findings suggest that there is an optimal level of D2 activation for cognition, since both reducing and increasing D2 receptor activation can induce deficits in perseveration and reversal learning. In these experiments, the 0.25mg dose of HAL was not sufficient to improve reversal learning when OVX rats received no E2 replacement. The low E2 replacement regimen facilitated the 0.25mg dose of HAL, reducing transmission at the D2 receptor to an optimal level causing a decrease in perseveration and an improvement in reversal learning. In the high E2 replacement group, the

combination of a 0.25mg dose of HAL and high E2 reduced transmission at the D2 receptor excessively, resulting in no improvement in perseveration or reversal learning. This hypothesis is supported by our experiment with 0.13mg HAL; this dose of HAL was not sufficient to affect reversal learning in rats administered no and low E2 replacement, but there was a trend towards more rapid reversal learning in rats administered high E2 replacement. Although this difference did not reach significance, the effect size indicated that this was a large effect (Cohen's d = 0.82), and the pattern of behavior in high E2 replacement rats receiving 0.13mg HAL is opposite to that observed in high E2 rats receiving 0.25mg HAL (see Fig 3). This suggests that the 0.25mg dose of HAL was optimal for AMPH sensitized rats administered low E2 replacement, but was excessive when administered in conjunction with the high E2 replacement regime, while the 0.13mg dose only had an effect when rats were also administered high E2 replacement. Clinical data corroborate this finding, as antipsychotic medications are shown to be more effective when administered in conjunction with E2 (Akhondzadeh et al., 2003; Kulkarni et al., 2014; Kulkarni, Gavrilidis, et al., 2012).

4.5 Mechanism for the interaction between E2 and HAL

Although the mechanisms through which E2 facilitates HAL are not fully elucidated, there is evidence indicating that E2 affects dopamine and D2 receptor availability and function in the STR, which could provide an explanation for the interaction between E2 and HAL. Ovariectomy results in significant decreases in D2 receptor binding in the STR and NAc, which is prevented by E2 replacement, suggesting that estrogens maintain D2 receptor density in these regions (Landry, Levesque, & Di Paolo, 2002; Le Saux, Morissette, & Di Paolo, 2006). E2 also promotes changes in the D2 receptor that convert the receptor to a low affinity state (Levesque & Di Paolo, 1988); in this low affinity state the D2 receptor is not activated by dopamine binding, making it functionally inert (Seeman, 2006). These findings indicate that E2 replacement administered to rats in this experiment would increase the density of D2 receptors, increasing binding sites for HAL, and decrease the effects of dopamine binding at D2 receptors by converting receptors to their low affinity state. Alternatively, there is evidence that combined E2 and HAL treatment reduces dopamine levels in the NAc more than HAL alone (Madularu et al., 2014). This suggests that E2 may facilitate the effects of HAL by reducing dopamine availability, therefore reducing binding at dopamine receptors, including the D2 receptor.

4.6 Conclusions

The findings of these three experiments in this study suggest that E2 alone has no effect on reversal learning or perseveration, but that E2 facilitates the effects of HAL to reduce perseveration and improve reversal learning, in AMPH sensitized rats. However, the high E2 replacement combined with the 0.25mg dose of HAL did not improve perseveration or reversal learning, possibly because the combination of high E2 and 0.25mg HAL reduced transmission at the D2 receptor excessively, reducing activation of the D2 receptor to sub-optimal levels. These findings indicate that administration of E2 in conjunction with antipsychotic drugs improves the efficacy of these drugs, so that they ameliorate some cognitive deficits associated with schizophrenia. This suggests that the optimal dose of HAL, when administered chronically as it is to individuals with schizophrenia, may differ across the menstrual cycle in females. Since cognitive deficits of this disorder are difficult to treat, and are the best predictor of functional outcome, the findings of these experiments have implications for the treatment of schizophrenia.

CHAPTER 4:
MEMBRANE-ASSOCIATED ESTROGEN RECPETORS IN THE DORSAL STRIATUM

CHAPTER 4A:
ESTROGEN RECEPTORS ARE FOUND IN GLIA AND AT EXTRANUCLEAR NEURONAL SITES IN THE DORSAL STRIATUM OF FEMALE RATS: EVIDENCE FOR CHOLINERGIC BUT NOT DOPAMINERGIC CO-LOCALIZATION.

Anne Almey, Teresa A. Milner, and Wayne G. Brake

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Preface:

The previous two chapters describe a series of experiments which demonstrate that estrogens affect some dopamine-dependent cognitive processes and the response to a dopamine antagonist, HAL. The mechanism through which these effects might occur remained unclear, since previous research reported relatively low levels of ERs in the terminal regions of the mesocorticolimbic and nigrostriatal pathways. Research in the hippocampus suggested that electron microscopy had sufficient resolution to detect ERs that were not observed using light microscopy, including mERs. Consequently, a series of experiments, described in Chapters 4, 5, and 6, used single and dual immunolabelling techniques and electron microscopy to determine the ultrastructural localization of ERs in the STR, NAc, and PFC. The initial experiments, described in the following Chapter, 4A, examined the distribution of ERs, ERα, ERβ, and GPER1, in the STR, and determined the proportion of ERα and GPER1 localized to dopaminergic and cholinergic neurons.

Abstract:

Estrogens rapidly affect dopamine (DA) neurotransmission in the dorsal striatum (STR) and DA-related diseases such as Parkinson's disease and schizophrenia. How estrogens influence DA function remains unclear, in part, because the ultrastructural localization of ERs in the STR is not known. Light microscopic studies of the STR have suggested the presence of ERs. This experiment used electron microscopy to determine if these ERs are at extranuclear sites in the STR, providing evidence for a mechanism through which estrogen could rapidly affect dopamine transmission. The STR was labelled with antibodies for ERα, ERβ, and GPER1 to confirm whether these ERs were present in this brain area. Following this, the STR was dual labelled with antibodies for ERα or GPER1 and tyrosine hydroxylase (TH) or VAChT to determine whether ERs are localized to dopaminergic and/or cholinergic processes, respectively. Ultrastructural analysis revealed immunoreactivity for ERα, ERβ, and GPER1 exclusively at extranuclear sites throughout the STR. ERα-, ERβ- and GPER1 immunoreactive (IR) profiles are mostly frequently observed in axons and glial profiles, but are also localized to other neuronal profiles. Dual labeling revealed that ERα and GPER1 –IR profiles are not dopaminergic but are sometimes cholinergic. As these receptors are exclusively extranuclear in the STR, binding at these receptors likely has rapid effects on neurotransmission in this region.

1. Introduction

Estrogens affect dopamine-dependent behaviours such as response memory (Quinlan, Hussain, and Brake, 2008; Zurkovsky et al., 2007) and selective attention (Nofrey, Ben-Shahar, and Brake, 2008; Quinlan et al., 2010). They are also implicated in dopamine-related diseases such as Parkinson's disease (for review see Bourque, Dluzen, and Di Paolo, 2009) and schizophrenia (Seeman, 2004). Estrogens increase dopamine (DA) neurotransmission in the dorsal striatum (STR; a.k.a. caudate/putamen; Becker and Rudick, 1999), which may contribute to these effects. Yet, how estrogens influence DA neurotransmission remains unclear. Because it is believed that estrogens act through binding at ERs to influence DA function, it is important to examine the ultrastructural localization of these receptors in the STR.

The localization of ERs in the STR is of particular interest because estrogens modulate dopaminergic activity at various steps in neurotransmission in this brain area. Both natural increases in estrogens across the estrous cycle, and E2 replacement in OVX rats, attenuate DA reuptake in the STR (Becker and Rudick, 1999; Thompson, 1999), possibly by reducing the availability of the dopamine transporter (Watson, Alyea, Hawkins, Thomas, Cunningham, and Jakubas, 2006). Furthermore, chronic E2 treatment results in significant increases in DA D2 receptor binding in the STR (Landry, Levesque and Di Paolo, 2002; Le Saux, Morissette and Di Paolo, 2006), with no corresponding increase in D2 mRNA in the STR. These authors suggested that this indicates that E2-induced increases in D2 receptors occur through binding at membrane-associated receptors (Lammers, D'Souza, Qin, Lee, Yajima and Mourdain, 1999). Finally, systemic injections of E2 are associated with higher levels of amphetamine-induced DA release in the STR (Becker, 1990; Becker and Rudick, 1999). These E2-induced increases in DA release occur rapidly, which further supports the idea that estrogens act through binding at mERs in this region (Becker and Rudick, 1999).

Estrogens may affect DA transmission in the STR via binding at the classical ERs, ERα and ERβ, or the more recently discovered G-protein-coupled estrogen receptor 1 (GPER1), formerly known by its orphan receptor name, GPR30. Using *in situ* hybridization, Shughrue et al. (1997) examined the distribution of ERα and ERβ throughout the central nervous system of the female rat and found no evidence of mRNA for these receptors in the STR. However, mRNA for ERα and ERβ was found in the STR of female mice using real time – polymerase chain reaction (Küppers and Beyer, 1999), and limited nuclear immunolabelling for ERα and ERβ was

observed in the in the STR of adult female mice using light microscopy (Mitra et al., 2003). Immunohistochemical studies using light microscopy also have assessed GPER1 distribution in the brain, and have shown this receptor to be abundant in the STR (Brailoiu et al., 2007). Thus, light microscopy data indicate that GPER1 receptors are present in the STR, and ERα and ERβ also may be found there at very low levels. Establishing if and where these ERs are located on striatal neurons would contribute to our understanding of how estrogens affect dopaminergic activity in this region.

Three experiments were conducted using immunolabelling techniques and electron microscopy (EM) to examine the distribution of ERs in the STR. Experiment 1 was conducted to determine whether ERs are found in this brain area, and, if so, whether they are located on neurons or glia. Since these experiments revealed that ERα and GPER1 are in numerous axon terminals, experiment 2 used dual immunolabelling EM to determine if these ERs are co-localized with tyrosine hydroxylase (TH), a marker of dopaminergic terminals (Gerfen and Wilson, 1996). Moreover, acetylcholine (ACh) has modulatory effects on dopaminergic transmission in the STR (Threlfell and Cragg, 2011), and ERα has been localized to cholinergic terminals in the hippocampus (Towart et al., 2003) while GPER1 has been localized to cholinergic neurons in medial septum, nucleus basalis magnocellularis, and STR (Hammond, Nelson and Gibbs, 2010). Therefore, experiment 3 used dual labeling EM to determine whether ERα or GPER1 are localized to profiles containing VAChT, a marker of cholinergic neurons.

2. Materials and Methods

2.1 Animals

Adult female (225 - 250 g; approximately 60 days old; N = 6) Sprague Dawley rats from Charles River Laboratories (Wilmington, MA) were pair-housed with *ad libitum* access to food and water and with 12:12 light/dark cycles (lights on 0600 - 1800). All procedures were in accordance with the National Institutes of Health guidelines and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. The rats used in these experiments are the same as those used by Williams and colleagues (Williams, Torres-Reveron, Chapleau and Milner, 2011). After arrival, rats acclimated 1 week to the vivarium after which estrous cycle phase was determined using vaginal smear cytology (Marcondes, Bianchi and Tanno, 2002; Turner and Bagnara, 1971). Tissue from rats in the diestrus 2 phase of the estrous

cycle was analyzed for these experiments. Estrous phase was verified by measuring uterine weights and plasma E2 levels from blood samples (see Williams et al., 2011).

2.2 Antisera

ERα: A rabbit polyclonal antiserum (AS409) produced against almost the full peptide for the native rat ERα (aa 61 through the carboxyl terminus) was supplied by S. Hayashi. This antibody was previously tested for specificity, and shown to recognize both ligand bound and unbound receptors (Alves, Weiland, Hayashi and McEwen, 1998; Okamura, Yamamoto, Hayashi, Kuroiwa and Muramatsu, 1992). On immunoblots of uterine lysates, this antiserum recognizes one major band migrating at ~67kD (the molecular weight of ERα; Milner et al., 2001). Preadsorption of the antibody with purified ERα resulted in no detectable bands in any of these locations (Milner et al., 2001).

ERß: A rabbit polyclonal antiserum produced against a peptide sequence in the C-terminus (aa 468-485) of the mouse ERß protein was used (Z8P; Zymed Laboratories, San Francisco, CA; Shughrue and Merchenthaler, 2001). This antibody has been shown to be specific for ERß (~60kDa) using Western Blot analyses, double label with ERß-mRNA using *in situ* hybridization, preadsorption control and absence of labeling in fixed brain sections prepared from ERß knock-out mice (Cruetz and Kritzer, 2002; Shughrue and Merchenthaler, 2001).

GPER1: (**Two antisera were used)**

Experiment 1: An affinity purified rabbit polyclonal antiserum produced against the N-terminus extracellular domain of the human GPER1 receptor (LifeSpan BioSciences, Inc., Seattle, WA) was used in experiment 1. This antibody recognized GPER1-green fluorescent protein transfected COS7 cells, and showed identical patterns of labeling to an antibody generated against the C-terminus of the GPER1 protein (Revankar, Cimino, Sklar, Arteburn and Prossnitz, 2005).

Experiments 2 & 3: These experiments used a rabbit polyclonal antiserum generated against a synthetic peptide, CAVIPDSTEQSDVRFSSAV (Multiple Peptide Systems, San Diego, CA), derived from the C-terminus of the human GPER1 receptor (Filardo, Quinn, Bland and Frackelton, 2000). In Western blots, this affinity purified antibody specifically recognizes a 38-kD band that corresponds to the mature 351-amino acid GPER1 polypeptide and does not recognize ERα or ERβ (Filardo et al., 2000). In brains fixed with 4% paraformaldehyde,

immunoreactivity was greatly reduced when the antibody was preadsorbed with 10mg/ml of purified C-terminal peptide (Hammond and Gibbs, 2011).

Vesicular acetylcholine transporter (VAChT): A goat polyclonal antiserum against the C-terminal synthetic peptide sequence corresponding to amino-acids 511-530 of the rat VAChT (Arvidsson, Riedl, Elde and Meister, 1997; Gilmor et al., 1996). This antibody was obtained commercially from Incstar (Stillwater, MN; now Millipore) and has been used in previous studies using identical labeling conditions (Threlfell and Cragg, 2011).

Tyrosine hydroxylase (TH): A mouse monoclonal antiserum against the full length of the peptide TH in the rat (Immunostar, Inc., Hudson, WI) was used. This antibody has been characterized extensively in fixed rat brain (Wang et al., 2006).

2.3 Tissue preparation

Rats were deeply anesthetised with sodium pentobarbital (150mg/kg, i.p.) and were perfused through the ascending aorta sequentially with: 10ml heparin (1000 U/ml) in saline; 50ml of 3.75% acrolein (Polysciences, Washington, PA) in 2% paraformaldehyde and 0.1 M phosphate buffer (PB; pH 7.4), and 200ml of 2% paraformaldehyde in PB. Brains were removed, cut into four 5mm blocks, and postfixed in 2% paraformaldehyde in PB for 30 minutes. The brains were sectioned coronally at 40µm thickness on a vibrating microtome (Vibratome; Leica) and stored in 30% sucrose and 30% ethylene glycol in PB (Milner, Waters, Robinson and Pierce, 2011) at -80°C.

Tissue sections containing the STR (Fig 1F) were rinsed in PB and coded with hole punches so that they could be pooled in single containers. Additionally, in single-labeling experiments for ERα, a section containing the ventromedial and arcuate nuclei of the hypothalamus was included in analyses; at the light microscopic level there is abundant ERα labeling in this region (Yaghmaie et al., 2010), so the success of immunolabelling could be confirmed at the light microscopic level prior to processing the tissue for electron microscopy. Similarly, in the experiment examining ERβ, a section containing the supraoptic nucleus was included, since previous light microscopy experiments have observed abundant imunolabelling for ERβ in this region (Shughrue et al., 1997). Sections were incubated in 1% sodium borohydride in PB for 30 minutes to remove any active aldehydes. Tissue then was rinsed in PB followed by 0.1M Tris-buffered saline (TBS; pH 7.6), and was incubated for 30 minutes in 1% bovine serum albumin (BSA) in TBS to reduce non-specific labeling.

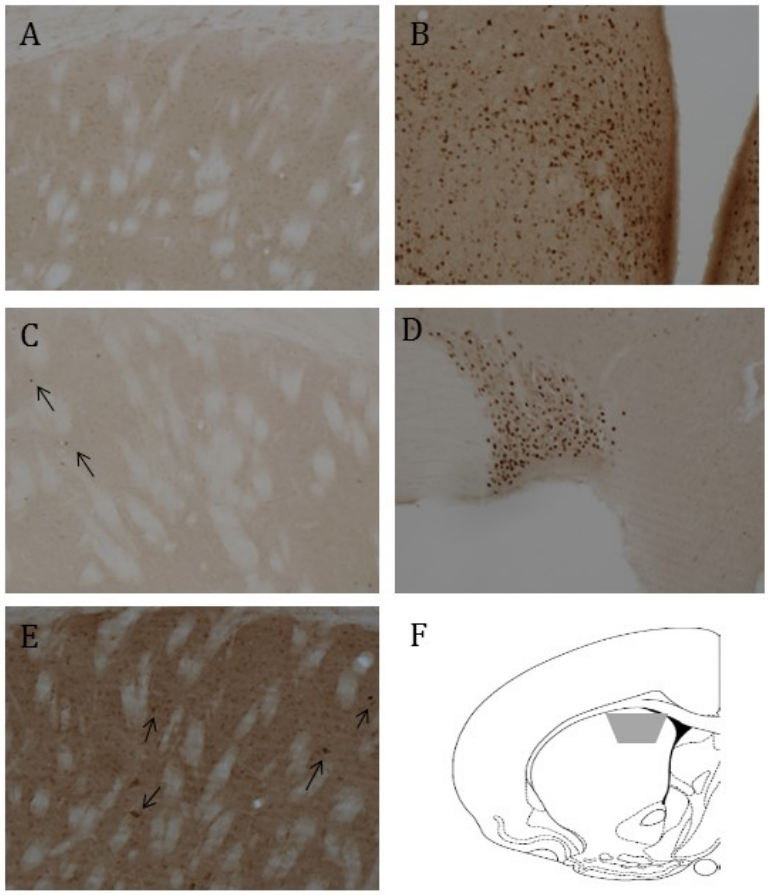

Figure 1. Light microscopic localization of ERs in the STR. **A)** Neither nuclear nor extranuclear ERα-IR is detected. **B)** Dense nuclear ERα-IR in the ventromedial and arcuate nuclei of the hypothalamus. **C)** No extranuclear ERβ-IR is detected; however, rarely a nucleus with ERβ-IR (arrow) is detected. **D)** Dense nuclear ERβ-IR in the supraoptic nucleus. **E)** Dense extranuclear GPER1-IR is detected in the neuropil; moreover, several cells with GPER1-IR (arrows) are seen. **F)** A coronal schematic of the striatum (atlas level 14; AP +1.00mm from bregma [Paxinos and Watson, 1998]) showing the region analysed by EM (grey trapezoid).

2.4 Immunohistochemical Labeling

Experiment 1. Free floating tissue sections containing the STR from 3 rats were processed for immunohistochemical localization of ERα, ERβ or GPER1. Tissue sections from each rat were incubated in anti-rabbit ERα (1:10,000 dilution), ERβ (1:2000 dilution) or GPER1 (Biosciences, 1:1000 dilution) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. ERs were visualized using the avidin-biotin complex (ABC) method (Milner et al., 2011). Briefly, the tissue was incubated in a 1:400 dilution of biotinylated donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.5% BSA in TBS for 30 minutes. Tissue was then incubated in peroxidase-avidin complex (Vector, Burlingame, CA) for a further 30 minutes, and 3,3-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and H_2O_2 in TBS for 6 minutes.

Experiments 2 and 3. Tissue from three rats was processed for immunohistochemical localization of ERα or GPER1 and TH or VAChT. Immunohistochemical procedures for ERs were identical to experiment 1 above. One day prior to processing either TH antisera (1:2000 dilution) or VAChT antisera (1:3000 dilution) was added to the diluent.

For immunohistochemical localization this experiment used pre-embedding dual labeling methods (Milner et al., 2011). The same ABC method described above for experiment 1 was used to visualize the ERs. TH and VAChT were detected using silver enhanced immunogold. Briefly, tissue sections were incubated for 2 hours in a 1:50 dilution donkey anti-mouse or donkey anti-goat IgG conjugated to 1-nm colloidal gold particles (Electron Microscopy Sciences [EMS], Fort Washington, PA) in 0.001% gelatin and 0.08% BSA in 0.01M phosphate buffered saline (PBS). Tissue sections then were rinsed in PBS, incubated in 1.25% glutaraldehyde in PBS for 10 minutes, rinsed again in PBS, followed by a brief wash in 0.2M sodium citrate (pH 7.4). A 7 minute Incubation in a silver solution (IntenSE; GE Healthcare) was used to enhance the conjugated gold particles.

2.4 Tissue fixation and embedding for ultrastructural analysis

Following immunolabelling, tissue sections from all three experiments were fixed for 60min in 2% osmium tetroxide in PB, dehydrated through a graded series of ethanols and propylene oxide, and embedded in EMbed 812 (EMS) between two sheets of Aclar (Milner et al., 2011). Ultrathin sections (~70nm thick) were taken through the dorsal region of the STR (Figure 1) using a Leica UCT ultratome. The tissue was collected on copper grids (EMS) and

then was counterstained using Reynolds' lead citrate and uranyl acetate. These grids were examined under a Philips CM10 electron microscope with an AMT digital camera. Final photomicrographs were generated from digital images, where brightness and contrast were adjusted using Windows Live Photo Gallery 2011. Adjusted images were assembled in Microsoft PowerPoint 2010.

2.5 Data Analysis

The subcellular distribution of ERα, ERβ, and GPER1 alone, and ERα and GPER1 co-localized with either TH or VAChT, were examined in the STR. A profile was considered positive for immunogold labeling if it contained two or more gold particles. Two STR sections of 54μm^2, from either the right or left hemispheres, were analyzed for each rat. For quantification analyses ER labeled profiles in each section were counted and categorized as: dendrites, dendritic spines, axons, axon terminals, or glia. The total number of labelled profiles was averaged for all six tissue sections (2 sections x 3 rats).The number of each type of single or dual labelled profile was divided by the total number of ER-IR profiles to determine the relative proportion of each type of labelled profile. Tissue selected for counting was taken from a depth of 0.2-1.5μm from the plastic–tissue interface, and only samples that were thin sectioned evenly across the plastic tissue interface were included in these analyses.

The type of neuronal profile was determined using the description of ultrastructural morphology from Peters et al. (Peters, Palay and Webster, 1991). Dendrites were large profiles (usually between 1.0 and 2.0 μm) that contained regular microtubule arrays and were sometimes contacted by terminals. Dendritic spines were small (usually between 0.3 and 0.4 μm), sometimes contained a spine apparatus or budded from dendritic shafts and formed synaptic contacts with axon terminals. Axon profiles were less than 0.2μm in diameter, contained a few small vesicles, and did not form synapses within the plane of section. Axon terminals had a cross-sectional diameter greater than 0.3μm and contained numerous synaptic vesicles, and sometimes formed synapses with other neuronal profiles. Glial profiles were recognized by their conformation to the boundaries of other profiles, and their lack of microtubules. Finally, soma were identified by their extremely large size, a lack of microtubules and high numbers of cellular organelles. All sections were assessed for nuclear labeling, however, soma were not included in the single label or TH quantification analyses because they frequently occupy more than half of the area counted for analysis, reducing the overall number of ER-IR profiles. Soma were

included in the analyses with VAChT, as high levels of colocalization were observed in these profiles, and we did not want to underestimate the colocalization between the ERs and VAChT. Contact between neuronal profiles refers to synapses identified by synaptic density, and appositions were defined as adjacent profiles that did not form a synapse in the plain of section.

3. Results

3.1 Experiment 1: Single labeling for ERs

By light microscopy, dense GPER1 but almost no ERα or ERß, is seen in the STR. By light microscopy, no nuclear or extranuclear labeling for ERα was observed in the STR (Fig. 1A). However, in the sections containing the ventromedial and arcuate regions of the hypothalamus, abundant ERα immunoreactive (IR) nuclei containing were seen indicating that immunolabelling for this receptor was successful (Fig. 1B). Similarly, no extranuclear ERß-IR profiles were observed although a rare ERß-IR nucleus was seen (Fig. 1C). However, many ERß-IR nuclei were seen in the supraoptic nucleus, confirming that labeling for this antibody was successful (Fig. 1D). In contrast to ERα or ERß, immunoreactivity for GPER1 was observed throughout the neuropil in the STR (Fig. 1E). GPER1 immunoreactivity was in the cytoplasm, but not the nuclei, of perikarya.

By EM, extranuclear ERα is observed in the STR. ERα immunoreactivity was present in all types of neuronal processes and glia in the STR (Table 1). Semiquantitative analysis demonstrated that 35% of ERα-IR profiles were axons, and 20% of ERα-IR profiles were axon terminals. In axons (<0.15 um in diameter), immunoreactivity was typically discrete and was affiliated with the plasma membrane or clusters of small vesicles (Fig. 2D). Axon terminals in the STR had cross sectional diameters that ranged from ~0.4-1.5µm, and contained numerous small synaptic vesicles (SSVs) and occasionally mitochondria, but no dense-core vesicles (Fig. 2A and C). ERα immunoreactivity was commonly found in clusters of reaction product around SSV (Fig. 2A and C) and was occasionally associated with the plasma membrane.

Peroxidase labeling for ERα was observed in neuronal perikarya, exclusively in the cytoplasm. This immunoreactivity was discrete and was usually associated with the plasma membrane or with mitochondria. Dendritic shafts accounted for ~9.4% of ERα-IR profiles and dendritic spines accounted for 8.3% of ERα-IR profiles. In the dendritic shafts, peroxidase reaction product was often affiliated with the mitochondrial and plasma membranes (Fig. 2B and

Table 1

ERα, ERβ, and GPER1 distribution in neuronal profiles and glia

Receptor		ERα	ERß	GPER1
Dendrites	**%**	9.4	13.2	18.7
	# SEM	10.6 ±1.7	2.3 ±0.6	17.3 ±3.8
Spines	**%**	8.3	1.9	9.3
	# SEM	8.3 ±0.9	0.3 ±0.6	10.1 ±1.7
Axons	**%**	35	49	36.4
	# SEM	32.3 ±3.4	8.7 ±1.1	33.6 ±4.7
Terminals	**%**	20.1	13.2	11.2
	# SEM	20.7 ±1.9	2.3 ±0.9	10.3 ±1.9
Glia	**%**	27.8	22.6	23.1
	# SEM	27.7 ±0.9	4 ±1.2	21.3 ±2.0
Total	**%**	100	100	100
	# SEM	99.7 ±4.9	17.7 ±2.7	92.3 ±12.3

The percentage of total IR profiles and number of IR profiles, and the corresponding standard error, observed in ~3000µm area of the dorsal striatum, averaged across rats.

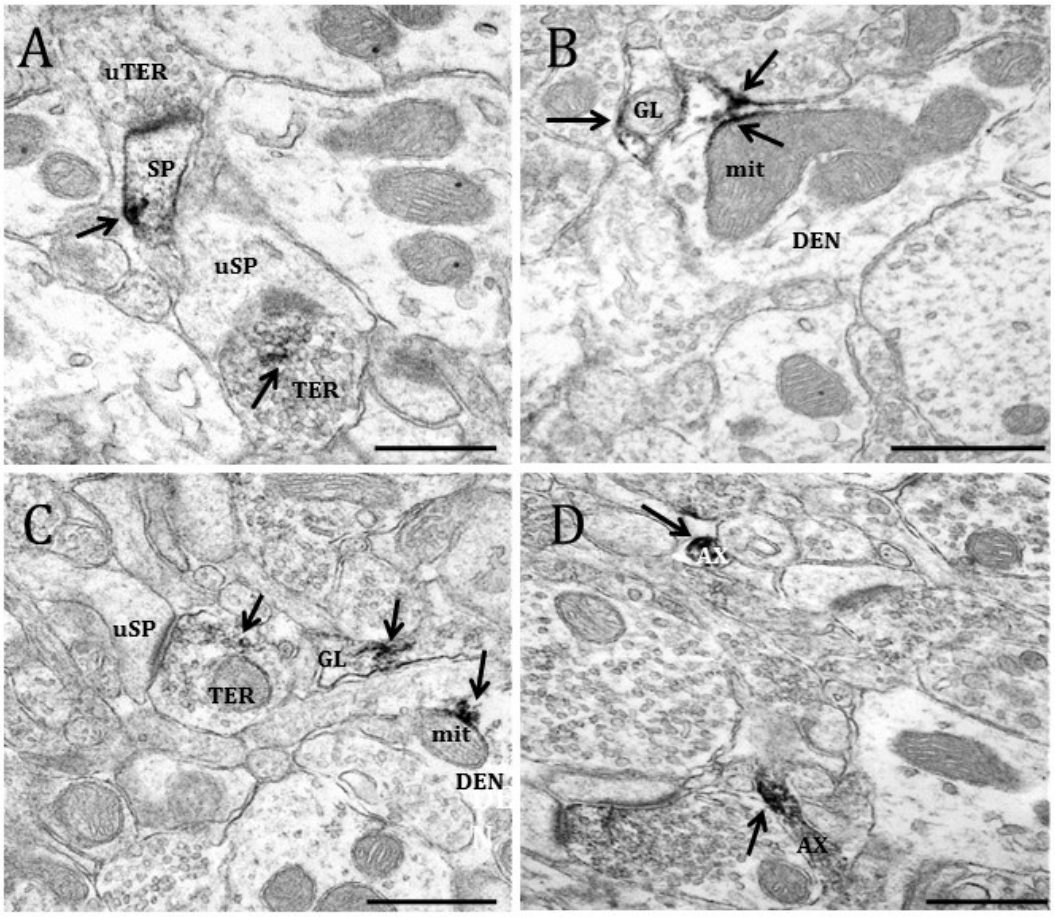

Figure 2. Electron micrographs show examples of ERα containing profiles. ERα-IR is observed in: **A)** a dendritic spine (SP) that is contacted by an unlabeled axon terminal (uTER), and an axon terminal (TER) that forms an asymmetric synapses with an unlabeled dendritic spine (uSP); **B)** a dendritic shaft (DEN) where it is affiliated with the plasma membrane and a mitochondria (mit), and in a glial process (GL); **C)** an axon terminal (TER) forming an asymmetric synapse with an unlabeled dendritic spine (uSP), a glial profile (GL), and on a mitochondria in a dendritic shaft (DEN); **D)** two unmyelinated axons (AX). In this and subsequent figures, labels are placed approximately in the center of the profile, while arrows point directly to immunoperoxidase/immunogold labeling. Black arrow = immunoperoxidase for ERα, bar = 500nm.

C), and microtubules. In dendritic spines, immunolabelling for ERα accumulated in the spine head, and was observed on the plasma membrane particularly near the post-synaptic density (Fig. 2A). ERα immunoreactivity was found at asymmetric synapses, where it was seen both pre- and post-synaptically. Occasionally, ERα-IR axon terminals synapsed onto ERα-IR spines. Finally, one quarter (27.8%) of ERα-IR was observed in glial cells of the STR. Labeling was primarily at the plasma membranes of glia (Fig. 2B).

By EM, extranuclear ERβ is observed in the STR. At the ultrastructural level ERß immunoreactivity was observed at extranuclear sites in some neuronal profiles and in glial cells in the STR. Although ERß-IR profiles are observed in the STR, the number of profiles labeled for ERβ was fivefold less than profiles for ERα or GPER1 (Table 1). ERß immunoreactivity was most commonly observed in axons, where it constituted 49% of the total ERß-IR profiles. In axons (<0.15 um in diameter), immunolabelling was discrete and was localized primarily to the plasma membrane, but was also affiliated with clusters of small vesicles (Fig. 3B and C). Immunoreactivity for ERß also was found in axon terminals, which accounted for ~13% of the total immunolabelling. ERß-IR axon terminals ranged from 0.3-0.6µm, and contained numerous SSVs and occasional mitochondria, but did not contain dense core vesicles. ERß immunoreactivity was found in clusters of reaction product associated with SSV and was sometimes affiliated with mitochondria (Fig. 3B).

ERß immunoreactivity was not observed in the perikarya of the STR. However, ERß-IR dendrites accounted for 13% of immunolabelling. ERß immunoreactivity was rarely observed in dendritic spines, accounting for 2% of ERß immunolabeling. In dendrites, immunoreactivity was typically associated with the plasma membrane or with mitochondria (Fig. 3A). Finally, ERß-IR glial cells also was frequently observed, making up 23% of the total immunolabeling. In glial cells, labeling was discrete and was localized primarily at the plasma membrane.

By EM, extrasynaptic GPER1 is observed in the STR. Immunoperoxidase labeling for GPER1 also was observed throughout the STR (Table 1). This labeling was associated with both neurons and glia, and was found exclusively at extranuclear sites. Like ERα and ERß, most GPER1-IR profiles were presynaptic; axons and axon terminals accounted for 36.4% and 11.2% of the GPER1 labelled profiles, respectively. GPER1-IR axons were small (<0.15µm) and almost

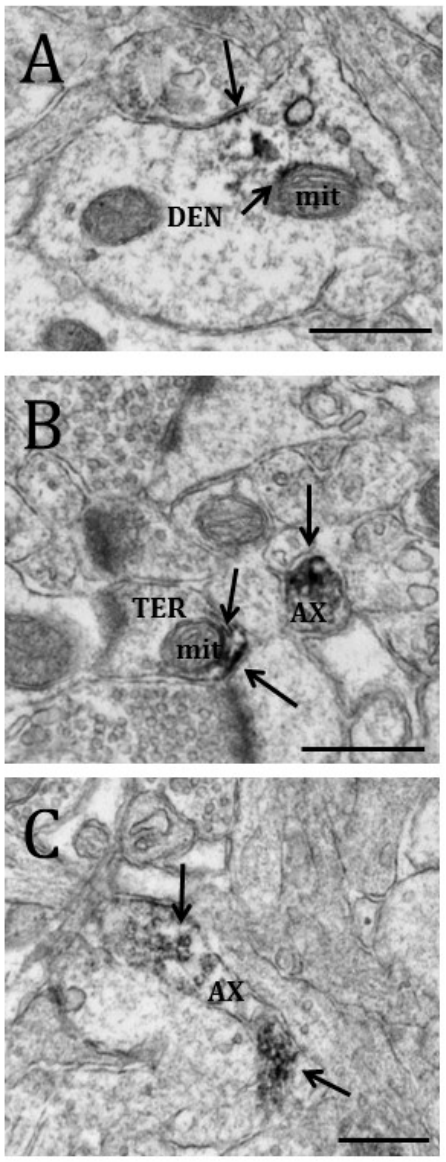

Figure 3. Electron micrographs show examples of profiles containing ERβ. Rarely**,** ERβ immunoreactivity was detected in: **A)** a dendritic shaft (DEN) and **B)** an axon terminal (TER). Within both profiles, ERβ immunoreactivity associated with mitochondria (mit). **C)** ERβ-IR was observed in an unmyelinated axons (AX). Black arrow = immunoperoxidase for ERβ, bar = 500nm.

always unmyelinated. The labeling in axonal profiles was usually discrete, and often associated with small clusters of vesicles (Fig. 4C). GPER1-IR axon terminals ranged from 0.3-0.6µm, and contained numerous SSVs, occasional mitochondria, but did not contain dense core vesicles. GPER1 labeling in terminals was most commonly clustered on groups of SSVs or the plasma membrane (Fig 4C).

GPER1 immunoreactivity was observed in neuronal perikarya exclusively in the cytoplasm; it was discrete, and was affiliated with Golgi apparati, mitochondria, and the plasma membrane (Fig. 4A). GPER1 immunoreactivity was also observed in dendritic shafts, where it constituted 18.7% of total GPER1 labelled profiles, and in dendritic spines, where it constituted 9.3% of the total profiles. In the dendritic shafts, GPER1 was typically associated with the plasma membrane, but also was affiliated with microtubules, mitochondrial membranes, and Golgi apparati (Fig. 4B). In dendritic spines, GPER1 peroxidase reaction product accumulated in the spine head, and was associated with the plasma membrane, particularly near the post-synaptic density (Fig. 4B-D). Although GPER1-IR was observed both pre and post-synaptically, it was rare for GPER1-IR terminals to synapse onto GPER1-IR spines. Finally, 23.1% of GPER1-IR was observed in glia in the STR; the labeling in glial cells was discrete, and was observed at the plasma membrane (Fig. 4D).

The total proportion of ERα-IR and GPER1-IR profiles were very similar in the STR. However a higher proportion of GPER1-IR profiles were dendrites, and a greater proportion of ERα-IR profiles were axon terminal (see Table 1).

3.2 Experiment 2: Dual labeling for ERs and TH

In dual labelled sections, immunoreactivity for both ERα and GPER1 had a similar distribution to that seen in experiment 1. In agreement with previous studies (Pickel and Chan, 1990), TH-IR profiles were also observed throughout the STR, exclusively in axons and axon terminals. These TH-IR terminals were 0.4-1.5µm diameter and contained numerous closely packed round SSVs; these terminals typically formed symmetric synapses with dendrites and occasionally perikarya. TH immunoreactivity was also infrequently observed in unmyelinated axons (0.1-0.15µm diameter). Although immunoreactivity for TH, ERα, and GPER1 were observed individually throughout the STR, immunolabelling ERα or GPER1 were rarely, if ever, observed in TH-IR profiles (see Table 2). TH-IR axons and terminals were often found in close

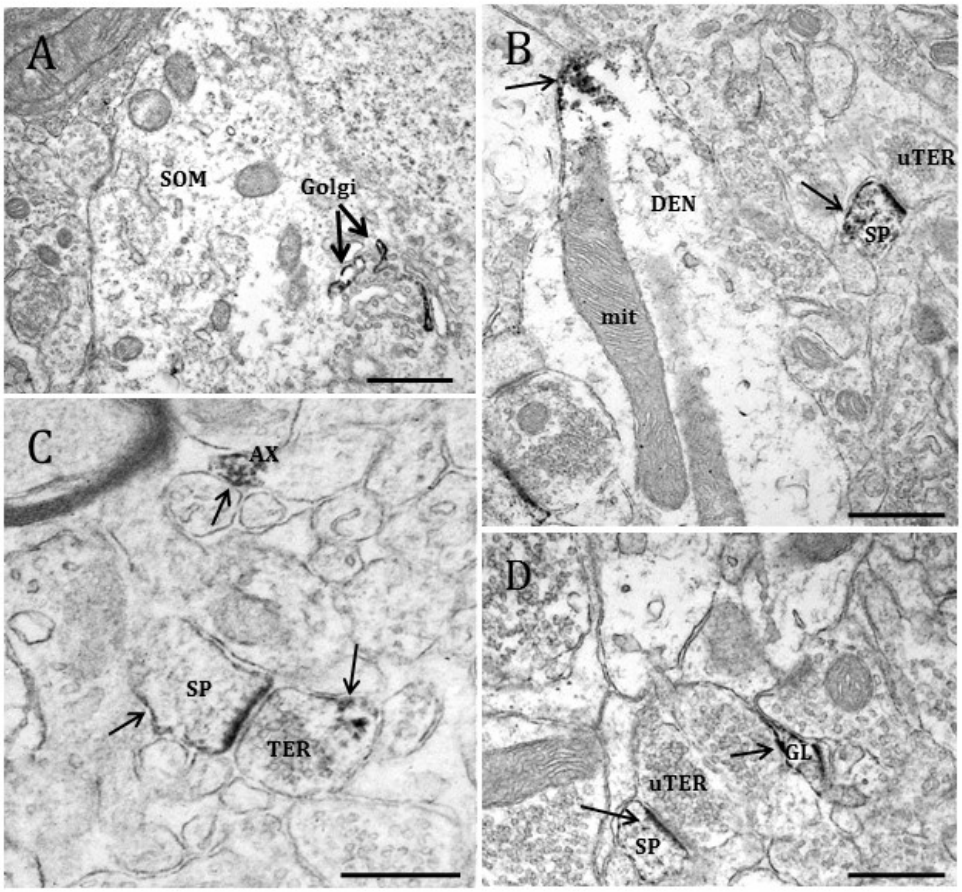

Figure 4. Electron micrographs showing examples of GPER1 containing profiles. GPER1-IR is localized to: **A)** Golgi bodies (Golgi) in a soma (SOM), **B)** a dendritic shaft (DEN) at the plasma membrane, and in a dendritic spine (SP) forming an asymmetric synapse with an unlabeled axon terminal (uTER) **C)** an unmyelinated axon (AX) and an axon terminal (TER) forming an asymmetric synapse with a dendritic spine (SP); **D)** a glial process (GL) and dendritic spine (SP) contacted by an unlabeled terminal (uTER). Black arrow = immunoperoxidase for GPER1, bar = 500nm.

3.3 Experiment 3: Dual labeling for ERs and VAChT

VAChT labeling was observed in multiple types of profiles, including axon terminals, dendrites, and perikarya, which is in agreement with previous studies examining cholinergic neurons in the STR (Pickel and Chan, 1990; Threlfell and Cragg, 2011). In axon terminals, VAChT-IR was associated with the membranes of SSVs (Fig. 5D, 6A). VAChT-IR was scattered throughout dendrites and soma, sometimes affiliated with microtubules and the endoplasmic reticulum (Fig. 5A- C, 6 A-C). A low proportion of ERα-IR was observed in VAChT-containing profiles, with the greatest proportion of co-localization in dendrites and axon terminals (see Table 3, Fig. 5A and D). Low levels of co-localization were observed between GPER1 and VAChT-IR, primarily in dendrites and perikarya (see Table 3, Fig 6 B-D). GPER1-VAChT-IR profiles were observed twice as frequently as ERα-VAChT-IR profiles**.** In rare instances VAChT-IR profiles were observed in apposition to either ERα-IR or GPER1-IR profiles (Fig. 6B).

4. Discussion

These experiments demonstrated that at the ultrastructural level, ERα, ERβ, and GPER1-IR is localized exclusively to extranuclear sites in both neuronal and glial profiles in the STR of female rats. Labeling for ERα and GPER1 is not detected in dopaminergic terminals, but is found in a small proportion of cholinergic interneurons.

4.1 Methodological Considerations

To determine whether ERα, ERβ, and GPER1 are found in the STR, and to localize ERα or GPER1 to TH or VAChT containing neurons, the present study used both immunoperoxidase and immunogold labels and preembedding methods. The ERα antibody and the two GPER1 antibodies had similar cellular and subcellular localizations when observed with EM, increasing confidence in the accuracy of these findings. Additionally, in accordance with previous research (Pickel and Chan, 1990), TH labeling is restricted to axons and terminals of the STR, while VAChT is seen in all neuronal profiles.

The preembedding EM immunohistochemical methods used in these experiments have been shown to result in excellent cellular morphology and allows for discrete subcellular localization of antigens (Leranth and Pickel, 1989). To ensure that any differences in number of labelled profiles were not due to differences in antibody penetration or sample size, all tissue

Table 2

ERα and GPER1 distribution in profiles containing TH or VAChT

		ERα or GPER-1 + TH				ERα or GPER-1 + VAChT			
Receptor		ERα	GPER-1	ERα + TH	GPER-1 + TH	ERα	GPER-1	ERα + VAChT	GPER-1 + VAChT
Dendrites	%	18.8	18.1	-	-	24.7	27.3	4.8	13.2
	# ±SEM	13.3 ±2.3	10.2 ±1.4	-	-	19.7 ±4.9	20.2 ±1.2	1.0 ±0.4	2.6 ±0.2
Spines	%	8.4	9.4	-	-	7.8	5.2	2.6	4.3
	# ±SEM	6.0 ± 0.4	5.3 ±1.6	-	-	6.2 ± 0.6	3.8 ±1.3	0.2 ±0.2	0.2 ±0.2
Axons	%	40.1	44.4	0.7	2	30.4	33.1	-	2
	# ±SEM	28.3 ±7.0	25.0 ±2.3	0.2 ±0.2	0.7 ±0.3	24.2 ±6.5	24.5 ±2.9	-	0.5 ±0.4
Terminals	%	14.6	14.2	1.6	2.8	15.5	20.5	5.1	3.3
	# ±SEM	10.3 ±1.4	8.0 ±1.3	0.2 ±0.2	0.3 ±0.3	12.3 ±2.9	15.2 ±2.6	0.7 ±0.4	0.5 ±0.2
Glia	%	17.4	17.2	-	-	17.8	12.6	-	-
	# ±SEM	12.3 ±1.6	9.7 ±5.8	-	-	14.2 ±3.3	9.3 ±2.3	-	-
Soma	%	N/A	N/A	N/A	N/A	1.3	1.4	14.3	17
	# ±SEM	N/A	N/A	N/A	N/A	1.0 ±0.3	1.0 ±0.3	0.2 ±0.2	0.2 ±0.2
Total	%	99.4	97.1	0.6	1.7	97.5	94.1	2.5	5.9
	# ±SEM	70.7 ±11.6	56.3 ±6.1	0.4 ±0.4	1.0 ±0.6	77.5 ±17.7	74.0 ±6.4	2.0 ±1.2	4.7 ±1.0

The percentage of total IR profiles and number of IR profiles, and the corresponding standard error, observed in ~3000µm area of the dorsal STR, averaged across rats.

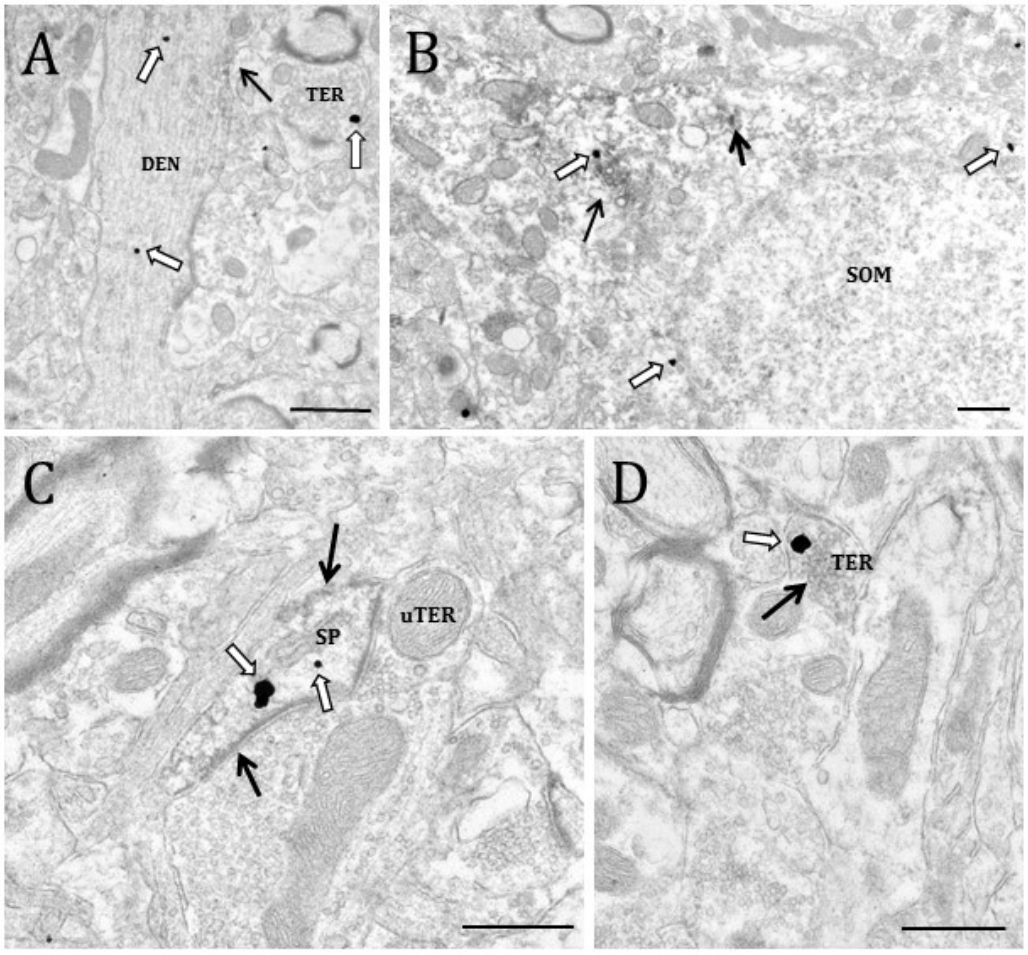

Figure 5. Electron micrographs show examples of profiles containing ERα and VAChT immunoreactivity. **A)** ERα localized to a VAChT-IR dendrite (DEN), and a VAChT positive terminal (TER). **B)** A soma (SOM) containing immunogold labeling for VAChT and immunoperoxidase labeling for ERα; **C)** a dendritic spine (SP) containing ERα and VAChT immunoreactivity that forms a synapse with an unlabeled axon terminal (uTER); **D)** an axon terminal (TER) containing both ERα and VAChT immunoreactivity. Black arrow = immunoperoxidase for ERα, White arrow = immunogold for VAChT, bar = 500nm.

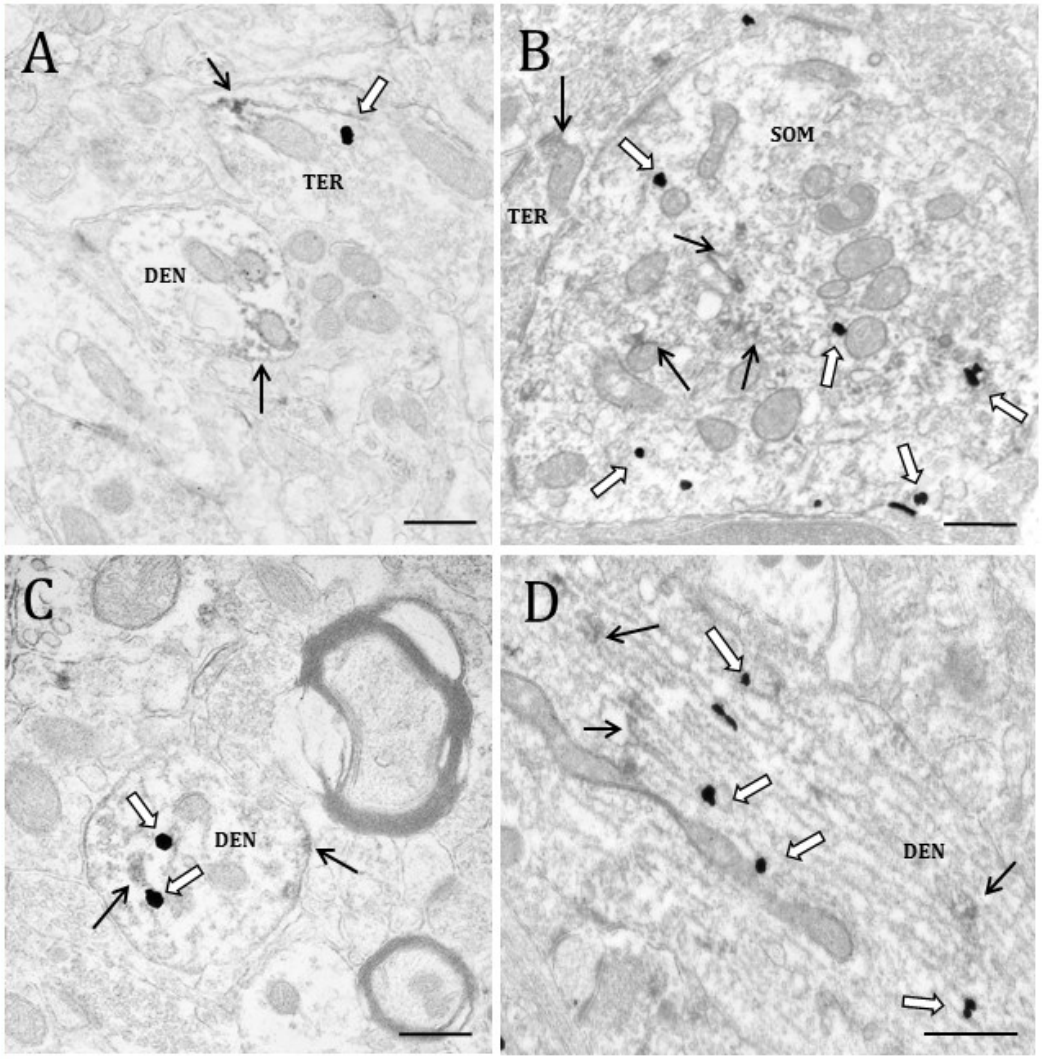

Figure 6. Electron micrographs show examples of GPER1 and VAChT containing profiles. **A)** GPER1 localized to a VAChT-IR terminal (TER), and a GPER1-IR dendrite (DEN). **B)** A soma (SOM) containing immunogold labeling for VAChT and immunoperoxidase labeling for GPER1. A GPER1-IR terminal (TER) is in apposition to the soma. **C)** A GPER1-IR and VAChT-IR dendrite (DEN); **D)** a large dendrite (DEN) containing immunoreactivity for both GPER1 and VAChT. Black arrow = immunoperoxidase for GPER1, White arrow = immunogold for VAChT, bar = 500nm.

samples analysed for quantification were identical in size and taken from near to the plastic/tissue interface. This methodology tends to underestimate the absolute number of peroxidase labelled profiles, and underestimates immunogold labeling to a greater extent, as immunogold is more limited in penetration (Leranth and Pickel, 1989). Immunoreactivity for ERα, ERβ and GPER1 are discrete, so the absence of ER labeling within cellular profiles does not demonstrate that these profiles lack ERs. This does not negatively impact the findings of these experiments, as the goal was to investigate whether these receptors were found in this region and the type of neurons where they were localized. However, the quantification analyses presented here are likely conservative values, underestimating the actual numbers of profiles containing these ERs, and the frequency with which ERα and GPER1 are localized to cholinergic profiles.

4.2 ERα is detected at extranuclear sites

At the ultrastructural level, the location and types of ERα-IR profiles containing in the STR were consistent in both single and dual-labelled tissue. Extranuclear ERα is observed in all types of neuronal profiles and glial cells. This finding contrasts previous light microscopic and in situ hybridization studies, which observed almost no ERα in the STR (Mitra et al., 2003; Shughrue et al., 1997). The discrepant findings in the present study and previous studies are likely because of the greater resolution of electron microscopy. In fact, in this experiment this discrepancy was also found, as light microscopy was not sufficient to observe ERα immunoreactivity, but EM allowed for the detection of discrete ERα-IR profiles in the STR.

The majority of ERα-IR profiles are axons, axon terminals and glia. The presence of ERα in axons may simply reflect the transportation of these receptors from the perikarya to the terminal, but binding at these receptors may also alter protein transport or the transduction of electrochemical signals (Cheung, 1990; Verdier, Lund, and Kolta, 2003). Additionally, these presynaptic receptors may be important in the local control of transmitter release, as estrogens have been shown to decrease GABA transmission in the STR (Hu, Watson, Kennedy and Becker, 2006). ERα immunoreactivity is observed exclusively at extranuclear sites in the STR, which is in congruence with previous findings that have localized this receptor to extranuclear sites in other brain regions, such as the hippocampus of rodents (Milner et al., 2001) and the PFC of rhesus monkeys (Wang, Hara, Janssen, Rapp and Morrison, 2010). Binding at these receptors on the plasma membrane could rapidly alter dopaminergic transmission in the STR, which

provides a possible mechanism for estrogens' rapid effects on transmission in this brain area (Becker and Rudick, 1999).

4.3 ERβ is detected at extranuclear sites

ERβ immunoreactivity was observed exclusively at extranuclear sites neuronal and glia in the STR when examined via EM. Similar to findings with ERα, ERβ was rarely observed at the light level, which parallels light microscopy experiments that did not observe this receptor in the STR (Mitra et al., 2003; Shughrue et al., 1997). Additionally, the number of profiles labeled for ERβ was five-fold less than that seen for ERα or GPER1. This likely contributes to the lack of detection of ERβ-IR by light microscopy in the STR (Mitra et al., 2003; Shughrue et al., 1997).

The highest proportion of ERβ immunoreactivity was observed in axons and glial cells. Like ERα, the presence of ERβ-IR axons could reflect receptors in transport or could suggest that ERβ has a role in conduction of electrochemical signals (Cheung, 1990; Verdier et al., 2003). However, the scarcity of ERβ-IR axon terminals suggests ERβ has a limited role in directly modulating synaptic transmission. The localization of ERβ exclusively to extranuclear sites in the STR agrees with previous studies in the hippocampus and rostral ventrolateral medulla (Milner et al., 2005; Wang et al., 2006). Binding at these membrane-associated ERβ receptors could contribute to estrogens rapid effects on dopaminergic transmission in the STR.

4.4 GPER1 is detected at extranuclear sites

GPER1-IR is seen throughout the STR, which agrees with previous light microscopic findings (Brailoiu et al., 2007). At the ultrastructural level, GPER1 is observed at the plasma membrane and in the cytoplasm of various neuronal profiles, corresponding to previous research examining the distribution of GPER1 (Filardo et al., 2006; Filardo and Thomas, 2012; Matsuda et al., 2008). GPER1 is also observed at the plasma membrane of glial cells.

The highest proportion of GPER1 immunoreactivity is observed in dendrites and on glial cells. This suggests that binding at GPER1 in the STR is more likely to affect neurotransmission through post-synaptic mechanisms. Additionally, GPER1 is associated with Golgi apparati in the STR, similar to findings in hippocampus (Matsuda et al. 2006). However, in contrast to findings in COS7, HEC50, and CHO cell cultures (Otto et al., 2008), and the hippocampal formation (Funakoshi, Yanai, Shinoda, Kawano, and Mizukami, 2006; Matsuda et al., 2008), GPER1-IR was not associated with the endoplasmic reticulum in the STR. It was hypothesized that

regulatory steps in the biosynthesis of this protein occur at the endoplasmic reticulum (Filardo and Thomas, 2012), which would imply that GPER1 should be present at this site in the STR. It is unclear why GPER1 was not observed at this organelle in these experiments.

4.5 Extranuclear ERs are associated with mitochondria

ERα, ERβ, and GPER1 are all localized to mitochondrial membranes and to the plasma membrane of glial cells in the STR. Estrogens have been implicated in mitochondrial functioning and cellular metabolism (Araujo, Beyer, and Arnold, 2008; Razmara et al., 2008) and to our knowledge, this is the first time GPER1 have been observed on mitochondria. This provides a mechanism through which estrogens could affect mitochondrial functioning. Additionally, E2 is known to mediate glial-induced neuroprotection (Arevalo, Santos-Galindo, Bellini, Azcoitia, and Garcia-Segura, 2010; Liu et al., 2011) in part through binding at GPER1 (Liu et al., 2011). Thus, the localization of ERs to the plasma membrane of glia could contribute to the explanation of how estrogens are involved in glial-mediated neuroprotection.

4.6 Both ERα-IR and GPER1-IR are found in cholinergic, but not dopaminergic, profiles

Dopamine terminals in the STR predominantly have cell bodies originating in the substantia nigra pars compacta, although some axon collaterals originate from the ventral tegmental area. These DA terminals form synapses primarily with GABAergic medium spiny projection and interneurons (Gerfen and Wilson, 1996), but also interact with cholinergic interneurons (Threlfell and Cragg, 2011). Increases in systemic E2 have been consistently shown to increase DA availability in the STR (Becker, 1990; Becker, 1999; Becker and Rudick, 1999), and it was hypothesized that estrogens might have these effects through binding at receptors found on dopaminergic terminals in the STR. However, neither ERα nor GPER1-IR terminals were dopaminergic, insofar as they are not co-localized with TH. Consequently, this suggests that estrogens are acting at receptors on other neurons in the STR (e.g. cholinergic neurons), or at receptors in other brain regions, to elicit these effects. One potential alternate region where estrogens could be acting to affect STR DA transmission is the SN, as ERα and GPER1 have been localized in the SN (Brailoiu et al., 2007; Kuppers, Ivanova, Karolczak and Beyer, 2000). Moreover, estrogens can directly target dopaminergic neurons in the SN, which could alter DA release and reuptake in the STR (Becker and Beer, 1986; Kuppers et al. 2000). Previous studies that have found estrogen-induced effects on DA release and dopamine transporter functioning in the STR used systemic injections of E2 (Becker, 1990; Becker and Rudick, 1999; Watson et al.,

2006) and consequently estrogens could have been acting on receptors in the SN to have these effects on DA transmission in the STR. Further research is needed to determine whether estrogens effects on DA transmission in the STR result from estrogens binding in the SN.

Both ERα- and GPER1-IRs are localized to cholinergic profiles in the STR. The localization of ERα to cholinergic neurons agrees with findings in the hippocampus (Towart et al., 2003), and the localization of GPER1-IR to cholinergic neurons agrees with findings in the medial septum, nucleus basalis magnocellularis, and STR (Hammond et al, 2010). This finding suggests that estrogens could have rapid effects on cholinergic transmission by binding at extranuclear ERs on these neurons. Almost all profiles containing either ERα or GPER1 and VAChT are dendrites, indicating that estrogens binding at these receptors would affect post-synaptic cholinergic transmission. ACh has modulatory effects on dopaminergic activity in the STR (Threlfell and Cragg, 2011), so estrogen-induced changes in striatal cholinergic transmission could, theoretically, alter dopaminergic transmission in this brain region, providing an alternate mechanism for the rapid effects of estrogens on DA in the STR.

Less than 10% of ER labelled profiles are cholinergic. This could partially be due to our immunolabelling yielding conservative estimates of both the ERs and VAChT, but does imply that a large proportion of ER-IR in the STR is localized to an unknown neuron type. Over 95% of neurons in the STR are GABAergic interneurons and projection neurons (Gerfen and Wilson, 1996). Systemic injections of E2 rapidly reduce GABA concentrations in the STR (Hu et al., 2006) and antagonizing GABAergic neurons in the STR increases DA levels in this brain area (Adermark, Clarke, Erison and Soderpalm, 2011). These results indicate that estrogens alter GABAergic transmission in the STR which could indirectly alter DA transmission. Only GABA neurons and cholinergic interneurons have their soma and dendrites in the STR; ER-IR dendrites and soma that do not contain VAChT-IR are observed, so it is reasonable to hypothesize that these remaining ER labelled profiles are associated with GABA neurons or interneurons. Future research from our group will address whether ERα, ERβ and GPER1 are localized to GABA neurons in the STR.

4.7 Conclusions

These experiments demonstrate the presence of ERs in the STR with ERα and GPER1 predominating. All three receptors are localized exclusively to extranuclear sites, in various neuronal profiles and on glial cells, providing a mechanism through which estrogens could

rapidly alter transmission in the STR. ERα and GPER1 are not localized to DA processes in this brain area, but are found in a small proportion of ACh neurons. ACh has strong modulatory effects on DA transmission in the STR, so estrogens could indirectly affect DA transmission through altering cholinergic transmission.

CHAPTER 4B:
ERα AND GPER1 ARE LOCALIZED TO GABAERGIC NEURONS IN THE DORSAL STRIATUM

Anne Almey, Teresa A Milner, and Wayne G Brake

Preface:

The experiments described in Chapter 4A demonstrate that ERα, ERβ, and GPER1 are in the STR, located exclusively to extranuclear sites. These experiments also demonstrate that ERα and GPER1 are not localized to dopaminergic neurons in the striatum, but a low proportion of these receptors are localized to cholinergic neurons. Following these experiments the majority of ERs in the STR were localized to an unknown neuron type. GABAergic neurons are the most common type of neuron in the STR (Gerfen and Wilson, 1996) and research has demonstrated that estrogens rapidly decrease GABA availability in this region (Schultz et. al., 2009). Consequently, the experiment presented in Chapter 4B examines whether ERα and GPER1 are localized to GABAergic neurons in the STR, extending the finings presented in Chapter 4A.

Abstract:

Estrogens affect dopamine transmission in the STR, increasing dopamine availability, maintaining D2 receptor density, and reducing the availability of the dopamine transporter. Some of these effects of estrogens are rapid, suggesting that they are mediated by membrane associated receptors. Recently our group demonstrated that there is extra-nuclear labeling for ERα, ERβ, and GPER1 in the STR, but that ERα and GPER1 are not localized to dopaminergic neurons in this region. GABAergic neurons are the most common type of neuron in the striatum, and changes in GABA transmission affect dopamine transmission, so this experiment used electron microscopy to determine whether ERα or GPER1 were localized to GABAergic neurons. Tissue from the STR was labelled with antibodies for either ERα or GPER1 and an antibody for GABA to determine whether these receptors are localized to GABAergic neurons. Ultrastructural analysis revealed that ERα and GPER1 are observed in GABA neurons in the STR, exclusively at extra-nuclear sites. Colocalization of immunoreactivity for ERα/GPER1 and GABA was most commonly observed in GABAergic dendrites and axon terminals. These findings indicate that estrogens can rapidly affect GABA transmission in the STR, so estrogens could indirectly affect dopamine transmission via changes in GABA transmission.

1. Introduction

There is evidence that estrogens increase dopamine transmission in the dorsal STR. Increases in estrogens across the estrous cycle, and E2 replacement in OVX rats, attenuates dopamine reuptake in the STR (Becker, 1990b; Becker & Rudick, 1999; Thompson, 1999), possibly by reducing the availability of the dopamine transporter (Watson et al., 2006). Furthermore, chronic E2 treatment results in significant increases in dopamine D2 receptor binding in the STR (Landry et al., 2002; Le Saux et al., 2006), and systemic injections of E2 are associated with higher levels of amphetamine-induced dopamine release in the STR (Becker, 1990a; Becker & Rudick, 1999). These E2-induced increases in dopamine release occur rapidly, which suggests that estrogens act through binding at membrane-associated receptors in this region (Becker, 1990b; Becker & Rudick, 1999).

Until recently it was unclear how estrogens have these effects, since previous light microscopy and in situ hybridization studies observed very low levels of nuclear labeling for ERα and ERβ in the STR (Kuppers & Beyer, 1999; Shughrue, Lane, & Merchenthaler, 1999; Shughrue, Scrimo, & Merchenthaler, 1998). The newly discovered ER, GPER1, was observed in the perikarya of neurons in the STR (Brailoiu et al., 2007), but it remained unclear what types of neurons GPER1 was localized to. We used electron microscopy to examine the distribution of ERs in the STR (Almey, Filardo, Milner, & Brake, 2012). These experiments demonstrated that membrane-associated ERα and GPER1 are prevalent in the STR, and membrane associated ERβ is also present at lower levels. ERα, ERβ, and GPER1 were observed exclusively at extranuclear sites, and were localized predominantly to presynaptic profiles, either axons or axon terminals, suggesting that estrogens alter striatal transmission via presynaptic mechanisms (Almey et al., 2012). These mERs provide a mechanism for the rapid effects of estrogens in the STR. Because of the known effects of estrogens on dopamine transmission in the STR a dual labeling study examined whether ERs were localized to neuronal profiles containing TH, a marker for catecholaminergic neurons. This study observed no colocalization of immunoreactivity for ERα or GPER1 and TH, demonstrating that ERs are not localized to dopamine neurons in the STR (Almey et al., 2012). A second experiment was conducted examining cholinergic interneurons, identified using an immunolabel for VAChT, which demonstrated that ~10% of ERα and GPER1 -labelled profiles in the STR are cholinergic (Almey et al., 2012). Therefor a low proportion of

ERs are localized to cholinergic interneurons in the STR, but a large proportion of ER immunoreactivity in the STR is localized to an unknown neuron type.

The majority of neurons in the STR are GABAergic interneurons and projection neurons (Gerfen and Wilson, 1996). Systemic injections of E2 rapidly reduces GABA concentration in the STR (Hu, Watson, Kennedy, & Becker, 2006; Schultz et al., 2009), and antagonizing GABAergic neurons in the STR increases DA availability (Adermark, Clarke, Ericson, & Soderpalm, 2011). These results indicate that estrogens may alter GABAergic transmission in the STR which could indirectly alter DA transmission. It is reasonable to hypothesize that the remaining ER-labelled profiles are associated with GABA neurons. This experiment used electron microscopy and dual labeling for ERs and GABA to determine if ERs are localized to GABAergic neurons.

2. Method

2.1 Animals

Three adult female Sprague Dawley rats from Charles River Laboratories (Wilmington, MA), approximately 225-250g on arrival, were pair-housed with *ad libitum* access to food and water and with 12:12 light/dark cycles, with lights on at 6:00am. Tissue from rats in the diestrus phase of the estrous cycle was analyzed for experiments 1 and 2, and tissue from rats in the estrus phase of the cycle was used in experiment 3. All procedures were in accordance with the National Institutes of Health guidelines and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. The rats used in these experiments are the same as those used in previous experiments (Almey et al., 2012; Williams, Torres-Reveron, Chapleau, & Milner, 2011).

2.2 Antisera

For ERα identification, a rabbit polyclonal antiserum (AS409) produced against the full peptide for the native rat ERα was supplied by S. Hayashi. To visualize GPER1 this experiment used a rabbit polyclonal antiserum generated against a synthetic peptide, CAVIPDSTEQSDVRFSSAV (Multiple Peptide Systems, San Diego, CA) derived from the C-terminus of the human GPER1 receptor, which was supplied by E Filardo (Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). Details on specificity testing for these antibodies has been reported elsewhere (Almey et al., 2012). A third antibody, provided by A .Towle, was used for

identification of GABA. A rat polyclonal antiserum was produced against GABA-glutaraldehyde-hemocyanin conjugates, and was specificity tested using preabsorbtion with GABA-BSA, which eliminated immunoreactivity (Lauder, Han, Henderson, Verdoorn, & Towle, 1986). Additionally immunoreactivity of this antiserum has been reported to be consistent with the specificity of other GABA-antisera (Lauder et al., 1986).

2.3 Tissue preparation

Rats were perfused, and brains were prepared for immunolabelling of STR tissue as described previously (Almey et al., 2012; Milner, Waters, Robinson, and Pierce, 2011). Additionally, a tissue section containing the ventromedial and arcuate nuclei of the hypothalamus was included in the immunohistochemical procedure as a positive control. Abundant ERα labeling is present in this region (Kritzer, 2002), so the success of immunolabelling could be confirmed prior to processing the STR for EM.

2.4 Immunohistochemical labeling and tissue fixation and embedding.

Free floating tissue sections containing the STR (Fig 1A) from three rats were processed for immunohistochemical localization of ERα or GPER1. Briefly, sections were incubated in anti-rabbit ERα (1:10,000 dilution) or GPER1 (1:1000) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. One day prior to processing GABA antisera (1:2000 dilution) was added to the diluent. For immunoperoxidase labeling, sections were incubated in 1) biotinylated donkey anti-rabbit immunoglobulin (IgG; diluted 1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.5% BSA in TBS, 30 minutes; 2) avidin-biotin complex (Vector, Burlingame, CA), 30 minutes; and 3) 3,3-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and H_2O_2 in TBS, 6-7 minutes. For immunogold labeling sections were incubated in a 1:50 dilution donkey anti-rat conjugated to 1-nm colloidal gold particles (Electron Microscopy Sciences [EMS], Fort Washington, PA) and a 0.001% gelatin and 0.08% BSA in 0.01M

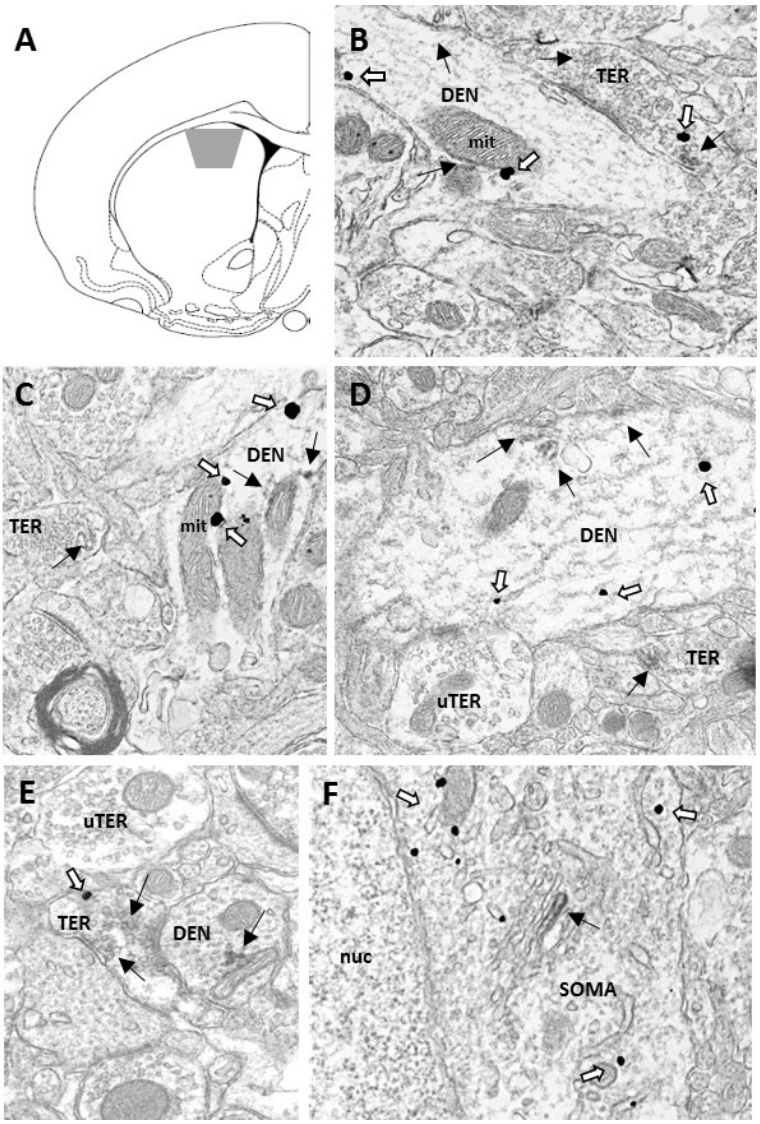

Figure 1. Electron micrographs demonstrating colocalization of ERα or GPER1 and GABA in the dorsal striatum. **A)** The area of the dorsal striatum examined in this experiment. **B)** A dendrite (DEN) containing immunogold labeling for GABA and immunoperoxidase labeling for ERα associated with the membrane and a mitochondrion forms a synapse with a GABA and ERα -IR terminal (TER). **C)** An ERα-IR and GABA-IR dendrite (DEN) that forms a synapse with an unlabeled terminal, and a terminal (TER) containing ERα-IR; **D)** A dendrite (DEN) containing GABA and GPER1 immunoreactivity associated with microtubules and the plasma membrane. **E)** A terminal (TER) containing GABA-IR and GPER1-IR associated with small synaptic vesicles, which forms a synapse with a GPER1-IR dendrite (DEN). **F)** A GABA-IR soma (SOM) containing GPER1 immunoreactivity that is associated with a Golgi body. Black arrow = immunoperoxidase for GPER1, White arrow = immunogold for VAChT, bar = 500nm.

phosphate buffered saline (PBS; in 1.25% glutaraldehyde in PBS) for 10 minutes. Sections were then rinsed in PBS, washed in 0.2M sodium citrate (pH 7.4), and incubated in a silver solution for ~7min (IntenSE; GE Healthcare) to enhance the conjugated gold particles. Following immunolabelling tissue sections were fixed in osmium, embedded in plastic, and sectioned and collected on grids, as described previously (Almey et al., 2012).

2.5 Analyses

Sections from the STR were examined under a Philips CM10 electron microscope with an AMT digital camera. The subcellular distribution of each ER was examined in two sections per rat; a 54μm^2 area of each section was counted in each section and categorized as: dendrites, dendritic spines, axons, axon terminals, or glia, using established criteria (Almey et al., 2012; Peters, 1991). The total number of labelled profiles were summed for the two sections, and averaged across the three rats. Tissue selected for analysis was taken from a depth of 0.2-1.5μm from the plastic–tissue interface, and only samples thin sectioned evenly across the plastic tissue interface were included in analyses. Soma were not included in the quantification analyses, as they frequently occupy more than half of the area analyzed, reducing the overall number of ER-IR profiles observed. Final photomicrographs were generated from digital images, where brightness and contrast were adjusted using GIMP 2.8. Figures were assembled in Microsoft PowerPoint 2013.

3. Results

The proportions of both ERα and GPER1 –IR profiles observed in the STR were comparable to those observed in the previous study (Almey et al., 2012). The majority of ERα and GPER1 immunoreactivity was observed at presynaptic sites, associated with axons (<0.15μm) or axon terminals (0.4-1.5μm; see Table 1); semi-quantitative analysis demonstrated that 36 % of ERα immunoreactivity was observed in axons and 32% was localized to axon terminals, while 40% of GPER1 immunoreactivity was localized to axons and 32% was localized to axon terminals. In ERα and GPER1 -IR axons were often observed at the membrane, and were also frequently observed in close proximity to SSVs. Immunolabelling for ERα and GPER1 was also observed at postsynaptic sites, in dendrites and dendritic spines. Labeling in dendrites accounted for 10% of ERα and 13% of GPER1 immunoreactivity, while labeling in spines accounted for 7% and 3 % of ERα and GPER1 immunoreactivity, respectively. Additionally,

Table 1

Distribution of ERα or GPER1 and GABA in the dorsal striatum.

Receptor		ERα	GPER1	Erα + GABA	GPER1 + GABA
Dendrites	**%**	10.8	13.0	35.3	44.1
	# SEM	28.3 ±0.3	34.0 ±1.7	10.0 ± 0.1	15.0 ±1.0
Spines	**%**	7.01	3.44	7.27	7.41
	# SEM	18.33 ±1.2	9.0 ±0.4	1.3 ±0.3	0.7 ±0.7
Axons	**%**	36.1	40.0	7.1	3.8
	# SEM	94.3 ±2.9	104.7 ±3.7	6.7 ±1.2	4.0 ±0.6
Terminals	**%**	32.5	32.1	27.1	30.2
	# SEM	85.0 ±0.6	84.0 ±9.9	23.0 ±3.1	25.3 ±3.3
Glia	**%**	13.2	11.3	4.8	14.6
	# SEM	34.7 ±5.5	29.7 ±5.7	1.7 ±0.7	4.3 ±1.9
Total	**%**	100	100	16.9	20.2
	# SEM	261.3 ±7.8	262.0 ±9.8	44.3 ±2.0	53.0 ±5.6

Percentage of total IR profiles and number of IR profiles, and the corresponding standard error, observed in ~6000μm area of the dorsal STR, averaged across rats.

13% of ERα immunoreactivity and 11% of GPER1 immunoreactivity was observed in glia. Both ERα and GPER1 were observed at mitochondrial membranes, again paralleling findings from the previous study in the STR (Almey et al., 2012). Immunogold labeling for GABA also paralleled previous findings, with GABA-IR commonly observed in terminals, dendrites, and perikarya of neurons in the STR (Delle Donne, Sesack, & Pickel, 1997; Gundersen, Ottersen, & Storm-Mathisen, 1996). GABA-IR axons and dendritic spines were also occasionally observed, but this was infrequent.

There are moderate levels of colocalization between ERα and GABA in the STR. Colocalization of ERα-IR and GABA-IR profiles were most commonly observed in dendritic shafts (Fig. 1 B and C), with 35.3% of ERα-IR dendrites containing GABA immunoreactivity (see Table 1). Colocalization between ERα was also frequently observed in axon terminals (Fig 1B); 27.1% of ERα-IR terminals also contained GABA immunoreactivity. Much lower levels of colocalization between ERα and GABA immunoreactivity were observed in axons and dendritic spines, with ~7% of ERα-IR axons and dendritic spines also containing GABA immunoreactivity. Glial profiles containing ERα and GABA immunoreactivity were also observed infrequently, with 4.8% of glia containing immunolabelling for ERα also containing GABA. Additionally, ERα immunoreactivity was observed in GABA-IR soma containing, although these profiles were not included in quantification analyses.

There were also moderate levels of colocalization between GPER1 and GABA in the STR. GPER1 immunoreactivity was most frequently colocalized with GABA immunoreactivity in dendritic shafts (Fig 1D); 40.1% of GPER1-IR dendritic shafts also contained GABA immunoreactivity (see Table 1). Colocalization was also observed in axon terminals (Fig 1E), with 30.2% of GPER1-IR axon terminals also contained GABA immunoreactivity. Similar to the findings with ERα, low levels of colocalization were observed between GABA and GPER1 in dendritic spines and axons. Specifically, 7.4% of GPER1-IR dendritic spines were GABAergic, and 3.8% of axons containing GPER1-IR were GABAergic. Colocalization was also observed in glia in the STR; 14.6% of GPER1-IR glia also contained GABA immunoreactivity. Finally, GPER1 immunoreactivity was observed in soma that were identified as GABAergic (Fig 1F), although these were not included in quantification analyses.

4. Discussion

Moderate proportion of both ERα and GPER1 were localized to GABAergic neurons in the STR, providing a mechanism for previous research demonstrating that estrogens rapidly decreased GABA availability in the STR (Hu et al., 2006; Schultz et al., 2009). The results of this study are similar to the results of our previous experiments examining ERs in the STR (Almey et al., 2012), increasing confidence in these findings. The greatest proportion of ERα and GPER1 colocalization with GABA was observed in dendritic shafts, and a substantial proportion of ERα and GPER1 –IR terminals were GABAergic. Additionally, GPER1 was localized to glial cells that also contained GABA immunoreactivity. There were low levels of colocalization between the ERs and GABA in axons and dendritic spines, likely due to the low levels of GABA immunoreactivity observed in these profiles. The findings of this experiment clearly demonstrate that ERα and GPER1 in the STR are localized to GABAergic neurons, where they are observed exclusively at extranuclear sites.

4.1 Methodological Considerations

Methodological considerations are discussed in detail in our previous publication (Almey et al., 2012). Briefly, the immunolabelling methods used here lead to excellent preservation of cellular morphology allowing for discrete localization of antigens (Leranth C., 1989). All tissue sections were identical in size and were taken near the plastic tissue interface to ensure that differences in antigen penetration did not affect the results of these experiments. Immunoreactivity for ERα and GPER1 is discrete, and a very thin plane of section is observed using EM, so a lack of immunoreactivity for ERs does not demonstrate that these profiles lack ERs. Additionally, for the dual-labeling analyses, the probability of detecting both immunomarkers in the same plane of section is decreased, particularly for small profiles. Consequently, the quantification analyses presented here are conservative, underestimating the number of ERs, and the frequency with which these receptors are localized to GABAergic profiles.

4.2 ERα and GPER1 are localized to GABAergic neurons in the NAc

Approximately one third of axon terminals that were IR for ERα and GPER1 were GABAergic. These ERs are positioned to directly affect transmitter release from these terminals, which corresponds to previous research that demonstrates that systemic injections of E2 rapidly (<30min) attenuate K^+-evoked GABA release in the STR (Hu et al., 2006; Schultz et al., 2009).

There is evidence that dopamine release in the STR is inhibited by GABA (Smolders, De Klippel, Sarre, Ebinger, & Michotte, 1995; Whitehead, Rose, & Jenner, 2001), thus E2-induced decreases in GABA could increase dopamine availability in the STR. More recently it was shown that E2-induced decreases in GABA affects dopamine dependent behaviour, providing evidence that E2 induced changes in GABA can affect dopamine-dependent behaviours (Shultz et al., 2009). The localization of ERα and GPER1 to GABAergic terminals provides a mechanism for estrogens effects on GABA transmission, and a means by which estrogens could indirectly affect dopaminergic transmission in the STR.

Additionally, approximately one third of the ERα and GPER1 –IR dendritic shafts were GABAergic. This suggests that estrogens can also affect postsynaptic transmission in GABAergic neurons of the STR. The localization of ERs to dendrites of GABAergic neurons is of particular interest because previous research has demonstrated that the majority of dopaminergic synapses in the STR are onto GABAergic medium spiny neurons (Pickel & Chan, 1990; Pickel, Towle, Joh, & Chan, 1988). Additionally, 70% of the dopamine synapses in the STR are onto dendritic shafts, likely dendrites of GABA neurons (Pickel et al., 1988). ERs localized to dendritic shafts of GABAergic neurons are in close proximity to dopamine-GABA synapses, ideally positioned to modify the dopamine-GABA interactions in the STR. This is a second potential mechanism by which binding at ERs could affect GABA transmission in the STR to indirectly alter dopaminergic transmission.

4.3 Conclusion

This experiment demonstrated that ERα and GPER1 are localized to GABA neurons in the STR. ERs were observed in GABAergic terminals and dendrites, suggesting that these ERs are positioned to modulate transmission at GABA synapses. These ERs on GABAergic profiles provide a mechanism for the rapid E2-induced decreases in GABA in the STR, and suggest that estrogens may indirectly alter dopaminergic transmission in this region by altering GABA transmission.

CHAPTER 5:
ESTROGEN RECEPTORS ARE OBSERVED AT EXTRANUCLEAR NEURONAL SITES AND IN GLIA IN THE NUCLEUS ACCUMBENS CORE AND SHELL: EVIDENCE FOR LOCALIZATION TO GABAERGIC AND CATECHOLAMINERGIC NEURONS

Anne Almey, Teresa A Milner, & Wayne G Brake

Preface:

Following the success of the electron microscopy studies examining the distribution of ERs in the STR, a second series of experiments were conducted examining the distribution of ERs in the NAc. These experiments, presented in Chapter 5, also determined what proportion of ERα and GPER1 are localized to catecholaminergic and GABAergic neurons. The NAc is subdivided into two regions, the core and the shell, which have anatomical and functional differences. Therefore, these experiments examined the distribution of ERs in the Core and Shell separately to determine whether ERs differed across these subregions of the NAc.

Abstract:

Estrogens affect dopamine dependent diseases/behaviour, and there is evidence that estrogens have rapid effects on dopamine release and dopamine D2 receptor availability in the NAc. Previous studies observe relatively low levels of nuclear labeling for ERα, ERβ, and GPER1 in the NAc, but these nuclear receptors cannot account for the rapid effects of estrogens in this region. Electron microscopy studies have demonstrated that there are membrane associated ERs in the STR, and these experiments extend these findings to the NAc Core and Shell. Immunolabelling techniques were used to determine whether ERα, ERβ, and GPER1 are localized to extranuclear sites in the NAc Core and Shell. These initial experiments were followed by dual labeling experiments that examined whether ERα and GPER1 were localized to catecholaminergic or GABAergic neurons in the NAc. Results of these experiments demonstrated that all three ERs were observed, almost exclusively, at extranuclear sites in the NAc, and that the distribution of these receptors was very similar in the Core and Shell subdivision. ERα, ERβ, and GPER1 were all observed primarily at presynaptic sites, in axons and axon terminals, suggesting that estrogens affect transmission in the NAc via presynaptic mechanisms. A small proportion of ERα and GPER1 are localized to catecholaminergic terminals, suggesting that binding at these ERs could alter release of catecholamines, including dopamine. A larger proportion of ERα and GPER1 are localized to GABAergic dendrites and terminals, suggesting that estrogens could alter GABAergic transmission to indirectly affect dopamine transmission in the NAc.

1. Introduction

There is evidence that estrogens affect the progression of dopamine-dependent diseases, protecting against some symptoms of schizophrenia (Kulkarni, Hayes, & Gavrilidis, 2012) and Parkinson's (Ragonese et al., 2004), and exacerbating the development of addiction to most drugs of abuse (Carroll, Lynch, Roth, Morgan, & Cosgrove, 2004). Additionally, estrogens also have effects on dopamine-dependent cognitive processes, including selective attention (Almey et al., 2013; Quinlan, Duncan, Loiselle, Graffe, & Brake, 2010), reversal learning (Arad & Weiner, 2012), and memory system bias (Almey et al. 2014; Quinlan et al., 2013; Quinlan, Hussain, & Brake, 2008; Zurkovsky, Brown, Boyd, Fell, & Korol, 2007), among other dopamine-dependent cognitive processes (for review see Luine, 2014). Maladaptive transmission in the STR and NAc is implicated in these diseases/disorders and cognitive processes (Gray et al., 1997; Howes & Kapur, 2009; Taghzouti, Louilot, Herman, Le Moal, & Simon, 1985), and it is hypothesized that estrogens alter dopamine transmission in these regions to elicit some of the effects on dopamine-dependent diseases and cognitive processes (Becker, 1999; Seeman and Lang, 1990; Thompson & Moss, 1997). To understand how estrogens affect transmission in the STR and NAc, a complete understanding of ER distribution in these regions is required.

A previous publication from our group examined the distribution of ERα, ERβ, and GPER1, in the STR, demonstrating that all three ERs observed at non-nuclear sites in this region (Almey et al., 2012); striatal ERs are not localized to dopaminergic neurons, but are localized to GABAergic interneurons (Almey, Milner & Brake, Chapter 4B) and to a lesser extent, cholinergic interneurons (Almey et al., 2012). This manuscript extends these findings by assessing the distribution of ERs in the NAc Shell and Core.

Estrogens modify dopamine activity at multiple stages of dopamine transmission in the NAc. First, systemic injections of E2 administered 48hours prior to testing resulted in significantly lower phasic dopamine release in the NAc (Thompson & Moss, 1994), but an infusion of E2 into the NAc increased phasic dopamine release within 15 minutes (Thompson & Moss, 1994). This suggests that there are opposing long-term and rapid effects of E2 on dopamine release in the NAc. E2 also rapidly increases the metabolism of dopamine, indicated by increased levels of DOPAC and HVA in the NAc within 30 minutes of E2 administration (Di Paolo, Rouillard, & Bedard, 1985). E2 replacement administered to OVX rats attenuates dopamine reuptake in the NAc (Thompson, 1999), providing a potential explanation for the E2-

induced increase in dopamine availability in the NAc. Additionally, ovariectomy results in decreases in D2 agonist and antagonist binding which is recovered by E2 replacement, suggesting that estrogens maintain levels of D2 receptors in the NAc (Landry et al., 2002; Le Saux et al., 2006). Some of these effects of E2 in the NAc occur over a long time scale (+24hours) suggesting that they are mediated by long-term effects of E2, while other effects of E2 occur rapidly (-30min).

Previous experiments using both light microscopy and in situ hybridization have examined the distribution of ERs in the NAc. Light microscopy and in situ hybridization studies observe low levels of immunolabelling for ERα and ERβ in the NAc, exclusively at nuclear sites (Mitra et al., 2003; Shughrue et al., 1998). GPER1 is also observed in soma in the NAc at low levels (Hazell et al., 2009), presumably localized to cellular organelles and the plasma membrane, as has previously been observed (Funakoshi, Yanai, Shinoda, Kawano, & Mizukami, 2006; Otto et al., 2008). Estrogens binding at nuclear ERα and ERβ, and GPER1 could be responsible for the long-term effects of estrogens in the NAc, and GPER1 could account for some of the rapid effects of estrogens in this region. However, the rapid effects of estrogens in the NAc could also be mediated by mERα or mERβ. There is evidence that light microscopy may not be sufficient to observe mERα and mERβ (Almey et al., 2012); ultrastructural analysis would determine whether mERα and mERβ, and GPER1, are localized to the NAc, and if so, what type of neuronal profiles these ERs are localized to.

These experiments used immunoelectron microscopy to examine the distribution of ERα, ERβ, and GPER1 in the NAc. There is evidence suggesting functional differences between two subregions of the NAc, the Core and the Shell (Ito & Hayen, 2011), so these experiments quantified ERs in these regions separately to determine if ER distribution differs in these subregions of the NAc. Initial experiments demonstrated that ERα and GPER1 are frequently observed at non-nuclear sites in the NAc, and ERβ is also observed at non-nuclear sites at lower levels. Following this experiment, dual labeling experiments were conducted to determine whether ERα and GPER1 were localized to catecholaminergic or GABAergic neurons in the NAc Core and Shell.

2. Method

2.1 Animals

Adult female (225 - 250 g; approximately 60 days old; N = 6) Sprague Dawley rats from Charles River Laboratories (Wilmington, MA) were pair-housed with *ad libitum* access to food and water and with 12:12 light/dark cycles (lights on 0600 - 1800). All procedures were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines and. The rats used in these experiments are the same as those used in our previous publication on mERs in the STR and by Williams and colleagues (Almey et al., 2012; Williams et al., 2011). After arrival, rats acclimatised to the animal colony for a week, and then estrous cycle phase was determined using vaginal smear cytology (Turner, 1971; Williams et al., 2011). Only female rats with two consecutive, regular, 4-5 day estrous cycles were included in the study. Tissue from rats in the diestrus 2 phase of the estrous cycle was analyzed for these experiments. Results of vaginal smear cytology, used to determine estrous cycle phase, were verified by measuring uterine weights and plasma E2 levels from blood samples collected during the perfusion procedure (Marcondes, Bianchi, & Tanno, 2002).

2.2 Antisera

ERα: A rabbit polyclonal antiserum (AS409) produced against almost the full peptide for the native rat ERα (aa 61 through the carboxyl terminus), was supplied by S. Hayashi. This antibody was previously tested for specificity, and recognizes both ligand bound and unbound receptors (Alves, Weiland, Hayashi, & McEwen, 1998; Okamura, Yamamoto, Hayashi, Kuroiwa, & Muramatsu, 1992). This antiserum recognizes one major band migrating at ~67kD (the molecular weight of ERα) on immunoblots of uterine lysates (Milner et al., 2001). When tested on immunoblots of ERα fusion protein, the AS409 antibody recognized minor bands migrating at ~110 kDa (likely the ERα/fusion protein complex), one major band migrating at ~67 kDa, and minor bands migrating at ~41-45 kDa (the degradation products of ERα, following the purification of ERα from the fusion protein). Preadsorption of the antibody with purified ERα resulted in no detectable bands in any of these locations (Milner et al., 2001).

GPER1: These experiment used a rabbit polyclonal antiserum generated against a synthetic peptide, CAVIPDSTEQSDVRFSSAV (Multiple Peptide Systems, San Diego, CA), derived from the C-terminus of the human GPER1 receptor (Revankar et al., 2005). In Western blots, this

affinity purified antibody specifically recognizes a 38-kD band that corresponds to the mature 351-amino acid GPER1 polypeptide and does not recognize either ERα or ERβ (Revankar et al., 2005). In brains fixed with 4% paraformaldehyde perfusion, immunoreactivity was greatly reduced when the antibody was preadsorbed with 10mg/ml of purified C-terminal peptide (Filardo, Quinn, Bland, & Frackelton, 2000).

ERß: a rabbit polyclonal antiserum produced against a peptide sequence in the C-terminus (aa 468-485) of the mouse ERß protein was used (Z8P; Zymed Laboratories, San Francisco, CA; (Shughrue & Merchenthaler, 2001). This antibody was specificity tested using Western Blot analyses, which demonstrated a single band migrating at ~60 kDa. Preadsorption of the antibody with purified ERß resulted in no detectable band at this location. Additionally, this antibody exclusively labelled profiles containing ERß-mRNA according to in situ hybridization (Creutz & Kritzer, 2002; Shughrue & Merchenthaler, 2001).

γ-Aminobutyric acid (GABA): A rat polyclonal antiserum was produced against GABA-glutaraldehyde-hemocyanin conjugates, and was specificity tested using preabsorbtion with GABA-BSA, eliminating GABA immunoreactivity (Lauder et al., 1986). Additionally, immunoreactivity of this antiserum is consistent with the specificity of other GABA-antisera (Lauder et al., 1986).

Tyrosine hydroxylase (TH): A mouse monoclonal antiserum against the full length of the peptide TH in the rat (Immunostar, Inc., Hudson, WI). This antibody has been characterized extensively in fixed rat brain (Pickel & Chan, 1990). The NAc has both dopaminergic and noradrenergic neurons (Kerfoot & Williams, 2011), so catecholaminergic profiles containing TH could be either dopamine or norepinephrine neurons.

2.3 Tissue preparation

Sodium pentobarbital was used to anesthetize rats (150mg/kg, i.p.). All rats were perfused through the ascending aorta with: 10ml heparin (1000 U/ml) in saline, followed by 50ml of 3.75% acrolein (Polysciences, Washington, PA) in 2% paraformaldehyde and 0.1 M phosphate buffer (PB; pH 7.4), and finally 200ml of 2% paraformaldehyde in PB. Rats' brains were removed, sectioned into four 5mm blocks, and postfixed in 2% paraformaldehyde in PB for 30 minutes. The brains were sectioned coronally at 40μm on a vibrating microtome (Vibratome; Leica) and stored in 30% sucrose and 30% ethylene glycol in PB at -80°C until immunohistochemical processing (Milner, Waters, Robinson, and Pierce, 2011).

Tissue sections containing the NAc Shell and Core (Fig 1A) were rinsed in PB and coded with hole punches so that they could be pooled in single containers. Additionally, a section containing the ventromedial and arcuate nuclei of the hypothalamus or the supraoptic nucleus was included in analyses for ERα and ERβ, respectively. Abundant ERα/ERβ labeling is observed in these regions using light microscopy (Shughrue et al., 1998; Yaghmaie et al., 2010), so the success of immunolabelling could be confirmed at the light microscopic level in hypothalamic tissue if no labeling was observed in the NAc. Sections were incubated in 1% sodium borohydride in PB for 30 minutes to remove any active aldehydes. Tissue then was rinsed in PB, followed by 0.1M Tris-buffered saline (TBS; pH 7.6), and was incubated for 30 minutes in 1% bovine serum albumin (BSA) in TBS to reduce non-specific labeling.

2.4 Immunohistochemical Labeling

Experiment 1. Free floating tissue sections containing the NAc Core and Shell from 3 rats were processed for immunohistochemical localization of ERα and GPER1. Tissue sections from each rat were incubated in anti-rabbit ERα (1:10,000 dilution) or GPER1 (Biosciences, 1:1000 dilution) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. Both ERs were visualized using the avidin-biotin complex (ABC) method (Milner, Waters, Robinson, and Pierce, J.P, 2011). Briefly, the tissue was incubated in a 1:400 dilution of biotinylated donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.5% BSA in TBS for 30 minutes. Tissue was then incubated in peroxidase-avidin complex (Vector, Burlingame, CA) for a further 30 minutes, and 3,3-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and H_2O_2 in TBS for 6 minutes.

Experiment 2. Immunohistochemical localization of ERα or GPER1 and TH or GABA was run on tissue from three rats. Tissue sections were incubated in either ERα antisera (1:10,000 dilution) or GPER1 antisera (Filardo; 1:1000 dilution) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. One day prior to processing either TH antisera (1:2000 dilution) or GABA antisera (1:2000 dilution) was added to the diluent.

For immunohistochemical localization this experiment used pre-embedding dual labeling methods (Milner, Waters, Robinson, and Pierce, 2011). The same ABC method described above for experiment 1 was used to visualize the ERs. TH and GABA were detected using silver enhanced immunogold. Briefly, tissue sections were incubated for 2 hours in a 1:50 dilution

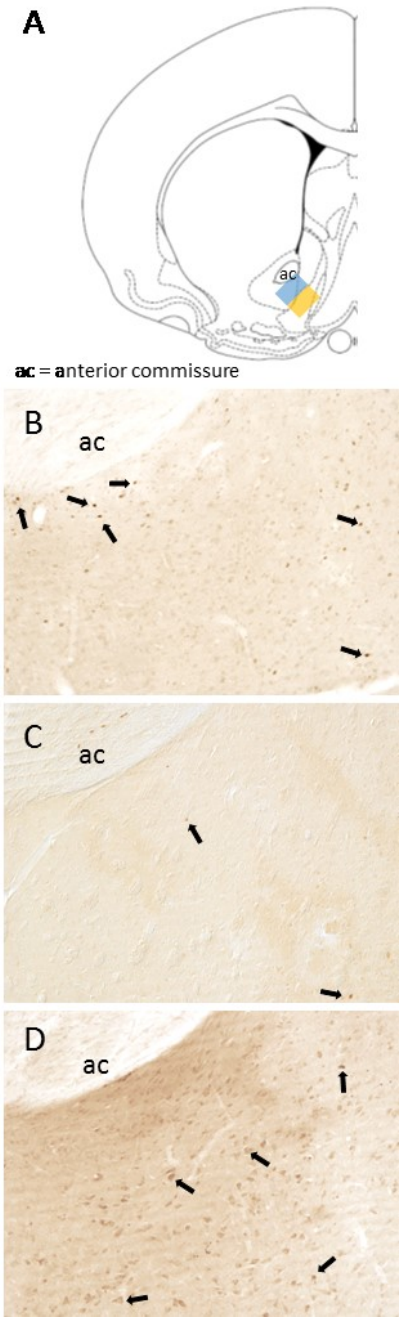

Figure 1. Light microscopic examination of ERs in the NAc. **A)** Depiction of the region analysed in electron microscopy experiments; the blue region was considered the NAc Core while the yellow was the NAc Shell. **B)** Moderate levels of nuclear, but no extranuclear, labeling were observed for ERα. **C)** Very sparse nuclear labeling for ERβ was observed. **D)** Dense extranuclear labeling, but no nuclear labeling for GPER1 was observed in the NAc. Black arrows = immunoreactive cells/nuclei.

donkey anti-rat or IgG conjugated to 1-nm colloidal gold particles (Electron Microscopy Sciences [EMS], Fort Washington, PA) in 0.001% gelatin and 0.08% BSA in 0.01M phosphate buffered saline (PBS). Tissue sections then were rinsed in PBS, incubated in 1.25% glutaraldehyde in PBS for 10 minutes, rinsed again in PBS, followed by a brief wash in 0.2M sodium citrate (pH 7.4). A ~7 minute Incubation in a silver solution (IntenSE; GE Healthcare) was used to enhance the conjugated gold particles.

Experiment 3. After the completion of experiments 1 and 2, a third pilot experiment was conducted to examine ERβ distribution in the NAc Core and Shell. Experiment 3 followed the procedures described for experiment 1, except that tissue was obtained from rats in the estrus phase of their cycle. Briefly, sections containing the NAc were incubated in anti-rabbit ERβ (1:2000 dilution) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. Following procedures from the previous experiments, ERβ was visualized using the avidin-biotin complex (ABC).

2.5 Tissue fixation and embedding for ultrastructural analysis

Following immunolabelling, tissue sections from all experiments were fixed for 60min in 2% osmium tetroxide in PB, dehydrated through a graded series of ethanols and propylene oxide, and embedded in EMbed 812 (EMS) between two sheets of Aclar (Milner, Waters, Robinson, and Pierce, 2011). Ultrathin sections (~70nm thick) including the NAc Shell and Core were taken (Fig 1A) using a Leica UCT ultratome. The tissue was collected on copper grids (EMS) and was counterstained using Reynolds' lead citrate and uranyl acetate. These grids were examined under a Philips CM10 electron microscope with an AMT digital camera. Final photomicrographs were generated from digital images, where brightness and contrast were adjusted using GIMP 2.8. Adjusted images were assembled in Microsoft PowerPoint 2010.

2.6 Data Analysis

The subcellular distribution of ERα, ERβ, and GPER1 alone, and ERα and GPER1 co-localized with either TH or GABA, were examined in the NAc Shell and Core. A profile was considered IR for immunogold labeling if it contained two or more gold particles. Two sections of 54μm^2 were analysed for the NAc Shell and the NAc Core, from either the right or left hemispheres, for each rat in all experiments. For quantification analyses ER labeled profiles in each section were counted and categorized as: dendrites, dendritic spines, axons, axon terminals, or glia. The total number of labelled profiles in the two 54μm^2 areas was calculated, and an

average was taken across the 3 rats. The number of each type of single or dual labelled profile was divided by the total number of profiles containing ER-IR to determine the relative proportion of each type of profile. Tissue selected for counting was taken from a depth of 0.2-1.5µm from the plastic–tissue interface, and only samples that were thin sectioned evenly across the plastic tissue interface were included in these analyses.

The type of neuronal profile was determined using the description of ultrastructural morphology from Peters et al. (Peters, Palay and Webster, 1991). Dendrites were large profiles (usually between 1.0 and 2.0 µm) that contained regular microtubule arrays and were sometimes contacted by terminals. Dendritic spines were small (usually between 0.3 and 0.4 µm), sometimes contained a spine apparatus or budded from dendritic shafts and formed synaptic contacts with axon terminals. Axon profiles were less than 0.2µm in diameter, contained a few small vesicles, and did not form synapses within the plane of section. Axon terminals had a cross-sectional diameter greater than 0.3µm and contained numerous synaptic vesicles, and sometimes formed synapses with other neuronal profiles. Glial profiles were recognized by their conformation to the boundaries of other profiles, and their lack of microtubules. Finally, soma were identified by their extremely large size, a lack of microtubules and high numbers of cellular organelles. All sections were assessed for nuclear labeling, but soma were not included in the quantification analyses, since they typically occupy approximately half of the area counted for analysis, reducing the overall number of ER IR profiles. Contact between neuronal profiles refers to symmetric and asymmetric synapses, and appositions. Asymmetric synapses were identified by their thicker postsynaptic density, while symmetric synapses had thin, equal pre- and post-synaptic densities. Appositions were any contact between profiles that was not a synapse, as indicated by the absence of synaptic density in the plane of section.

3. Results

3.3 Single labeling for ERs

By light microscopy, dense GPER1 and low levels of nuclear ERα or ERß, are localized to the NAc Core and Shell. Light microscopy observed moderate nuclear labeling for ERα (Fig 1B) and very low nuclear labeling for ERβ (Fig 1C) in the in the NAc. In contrast to ERα or ERß, GPER1-IR was observed throughout the neuropil of the NAc, but there was no nuclear labeling for GPER1 (Fig 1D).

By EM, extranuclear ERα is observed in the NAc Core and Shell. ERα immunoreactivity was observed in all types of neuronal processes and glia in the NAc Core and Shell (see Tables 1 and 2). Semiquantitative analyses showed that 35% of ERα-IR profiles in the NAc Core were axons, and 38% of ERα-IR profiles in the NAc Shell were axons (Figure 2D). ERα immunoreactivity was discrete and was affiliated with the plasma membrane and/or clusters of small vesicles in axons (<0.2 um in diameter). Analyses also demonstrated that 40% of ERα-IR profiles the NAc Core and 44% of ERα-IR profiles in the NAc Shell were in axon terminals (Figure 2A). Axon terminals in the NAc had cross sectional diameters that were ~0.3-1.5µm, and contained numerous small synaptic vesicles (SSV) and occasionally mitochondria. ERα immunoreactivity was commonly found in clusters of reaction product around SSV and was occasionally associated with the plasma membrane, sometimes in close proximity to synapses.

Peroxidase labeling for ERα was also observed at postsynaptic sites in the NAc. Dendritic shafts accounted for 7.8% of ERα-IR profiles in the NAc Core, and 5.7% of ERα-IR profiles in the NAc Shell (Figure 2C). Additionally, ERα immunoreactivity was also infrequently observed in dendritic spines; 5.5% of ERα-IR in the NAc Core, and 3.3% of ERα-IR in the NAc Shell was localized to dendritic spines (Figure 2D). In the dendritic shafts, peroxidase reaction product was often affiliated with the mitochondrial and plasma membranes, and microtubules. In dendritic spines, immunolabelling for ERα sometimes accumulated in the spine head, and was also observed on the plasma membrane, particularly near the post-synaptic density. ERα was frequently observed near asymmetric synapses, where it was seen both pre- and post-synaptically. Occasionally, ERα-IR axon terminals synapsed onto ERα-IR dendrites. Immunolabelling for ERα was sometimes observed in neuronal perikarya, associated with mitochondria or other cellular organelles, and sometimes associated with the plasma membrane. Lastly, 10.8% of ERα-IR in the NAc Core and 9.0% of ERα-IR in the NAc Shell were observed in glial cells (Figure 2B), primarily at the plasma membranes.

By EM, extranuclear ERβ is observed in the NAc Core and Shell. At the ultrastructural level ERß immunoreactivity was observed at extranuclear sites in all neuronal profiles and in glial cells in the NAc Core and Shell (see Tables 1 and 2). ERß immunoreactivity was most common in axons, where it constituted 49% of the total ERß-IR profiles in the NAc Core, and 45% of total ERβ- IR profiles in the NAc Shell (Figure 3C). In axons, immunolabeling was discrete and was

Table 1

ERα, ERβ, and GPER1 distribution in neuronal profiles and glia in the NAc Core

Receptor		ERα	ERß	GPER1
Dendrites	**%**	7.8	4.4	9.2
	# SEM	17.0 ±4.9	4.7 ±1.8	13.7 ±3.9
Spines	**%**	5.5	0.9	2.0
	# SEM	12.0 ±5.0	1.0 ±0.6	3.0 ±1.5
Axons	**%**	35.1	49.1	40.2
	# SEM	76.0 ±10.1	52.0 ±11.8	60.0 ±7.0
Terminals	**%**	40.5	39.0	33.0
	# SEM	87.7 ±12.4	41.3 ±4.1	49.3 ±1.3
Glia	**%**	10.9	6.6	15.6
	# SEM	23.7 ±3.2	7.0 ±1.0	23.3 ±2.9
Total	**%**	100	100	100
	# SEM	216.3 ±18.2	106.0 ±9.1	149.3 ±9.9

The percentage of total IR profiles and number of IR profiles, and the corresponding standard error, observed in ~6000µm area of the Nucleus Accumbens Core, averaged across rats.

Table 2

ERα, ERβ, and GPER1 distribution in neuronal profiles and glia in the NAc Shell

Receptor		ERα	ERß	GPER1
Dendrites	**%**	5.8	8.1	6.7
	# SEM	12.3 ±2.4	8.0 ±3.1	10.3 ±0.9
Spines	**%**	3.3	1.7	3.9
	# SEM	7.0 ±4.0	1.7 ±0.3	6.0 ±2.5
Axons	**%**	37.9	45.6	41.7
	# SEM	81.0 ±4.0	45.0 ±7.6	63.7 ±9.6
Terminals	**%**	44.1	36.5	34.1
	# SEM	94.3 ±3.5	36.0 ±2.9	53 ±6.4
Glia	**%**	9.0	8.1	13.5
	# SEM	19.3 ±2.2	8.0 ±1.7	20.7 ±1.5
Total	**%**	100	100	100
	# SEM	214.0 ±2.3	98.7 ±9.5	152.7 ±1.3

The percentage of total IR profiles and number of IR profiles, and the corresponding standard error, observed in ~6000µm area of the Nucleus Accumbens Shell, averaged across rats.

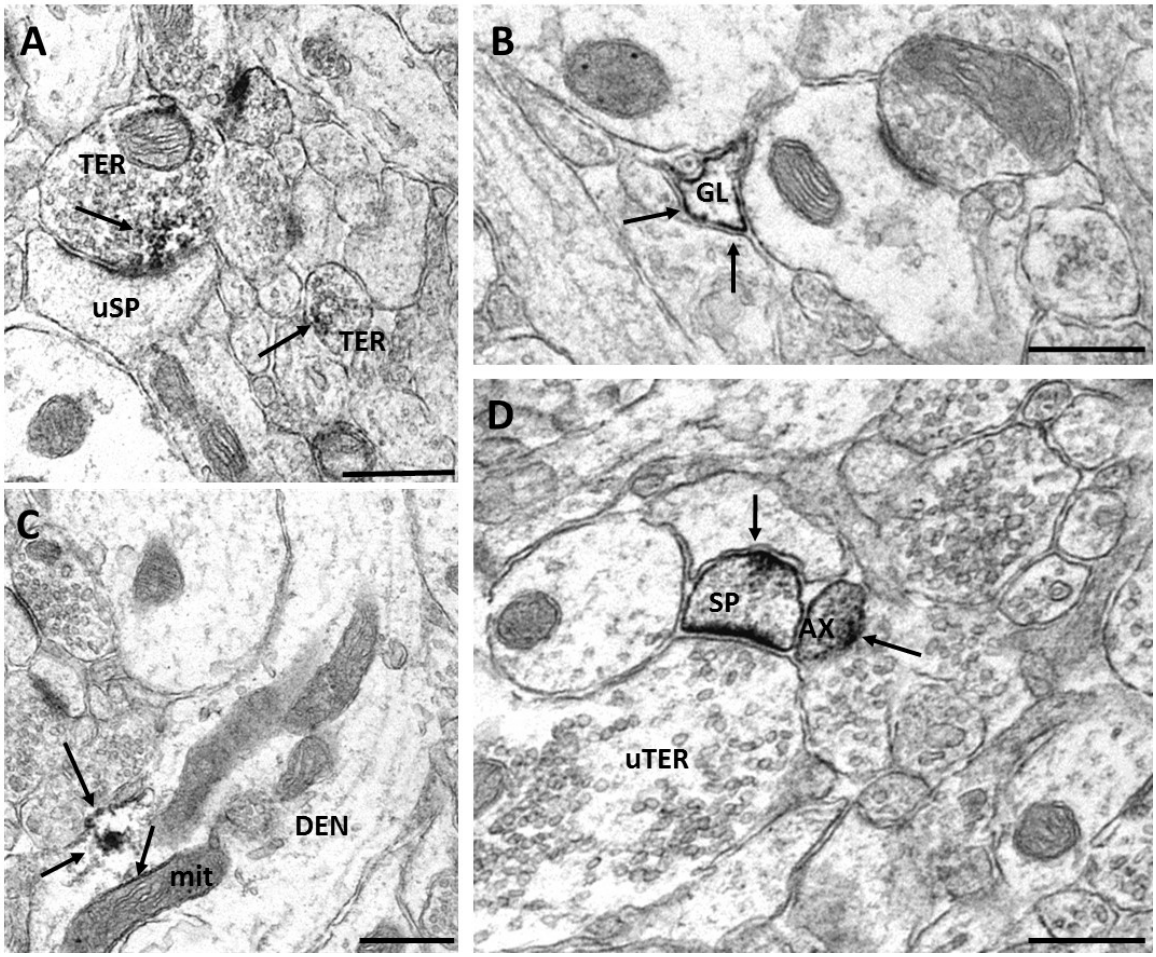

Figure 2. Electron micrographs show examples of ERα-containing profiles in the NAc Core and Shell. ERα-IR is observed in: **A)** a two axon terminals (TER), one that forms a synapse with an unlabeled dendritic spine (uSP), in the NAc Core; **B)** at the membrane of a glial process (GL) in the NAc Core; **C)** a dendrite (DEN), where it is associated with the membrane of a mitochondrion (mit), the cell membrane, and microtubules in the NAc Shell; **D)** a dendritic spine (SP) that forms a synapse with an unlabeled terminal (uTER), and an axon (AX) in the NAc Shell. *Black arrow*, Immunoperoxidase for ERα. Scale bar = 500 nm.

localized to the plasma membrane or clusters of small vesicles. ERß immunoreactivity also was found in axon terminals, which accounted for 39% of the total ERβ-IR profiles in the NAc Core and 36% of the total ERβ-IR profiles in the NAc Shell (Figure 3A). Axon terminals containing ERß immunoreactivity contained numerous SSVs and occasional mitochondria, but did not contain dense core vesicles. ERß immunoreactivity was found in clusters of reaction product associated with small synaptic vesicles and was sometimes affiliated with mitochondria and the plasma membrane.

ERß immunoreactivity was also observed infrequently at postsynaptic sites. Four percent of the total ERβ-IR profiles were dendrites in the NAc Core, and 9% of ERβ-IR profiles were dendrites in the NAc Shell (Figure 3B). ERβ immunoreactivity was almost never observed in dendritic spines, accounting for 0.9% of immunolabeling in the NAc Core and 1.7% of immunolabeling in the NAc Shell. In dendrites, immunoreactivity was typically associated with the plasma membrane or with mitochondria. ERß-IR perikarya were rarely observed in the NAc Core or Shell. Finally, ERß-IR glial cells were infrequently observed; 7% of the ERβ-IR profiles in the NAc Core and 8% of the ERβ-IR profiles in the NAc Shell were glia (Figure 3A). In glial cells, labeling was discrete and was localized primarily at the plasma membrane.

By EM, GPER1 is observed in the NAc Core and Shell. Immunoperoxidase labeling for GPER1 was also observed throughout both the NAc Core and Shell (see Tables 1 and 2). This labeling was associated with neurons and glia, and was found exclusively at extranuclear sites. Like ERα and ERß, most GPER1-IR profiles were presynaptic; GPER1-IR axons accounted for 40% of immunolabelling in the NAc Core and 42% of immunolabelling in the NAc Shell (Figure 4A and D). Axons containing GPER1-IR were small (<0.2µm) and almost always unmyelinated. The labeling in axonal profiles was usually discrete, and often associated with the membrane and small clusters of vesicles. GPER1-IR axon terminals accounted for 33% of the total GPER1 in the NAc Core and 34% of GPER1 in the NAc Shell (Figure 4C). Axon terminals containing GPER1 immunoreactivity ranged from 0.3-1.5µm, and contained numerous small synaptic vesicles and occasionally mitochondria, where labeling was frequently observed. GPER1 immunoreactivity was also occasionally observed in close proximity to synapses.

Low levels of GPER1 immunoreactivity were also observed in post synaptic profiles. GPER1-IR dendritic shafts constituted 9% of total GPER1-IR profiles in the NAc Core, and 7% of total GPER1-IR profiles in the NAc Shell (Figure 4C). There were low levels of GPER1

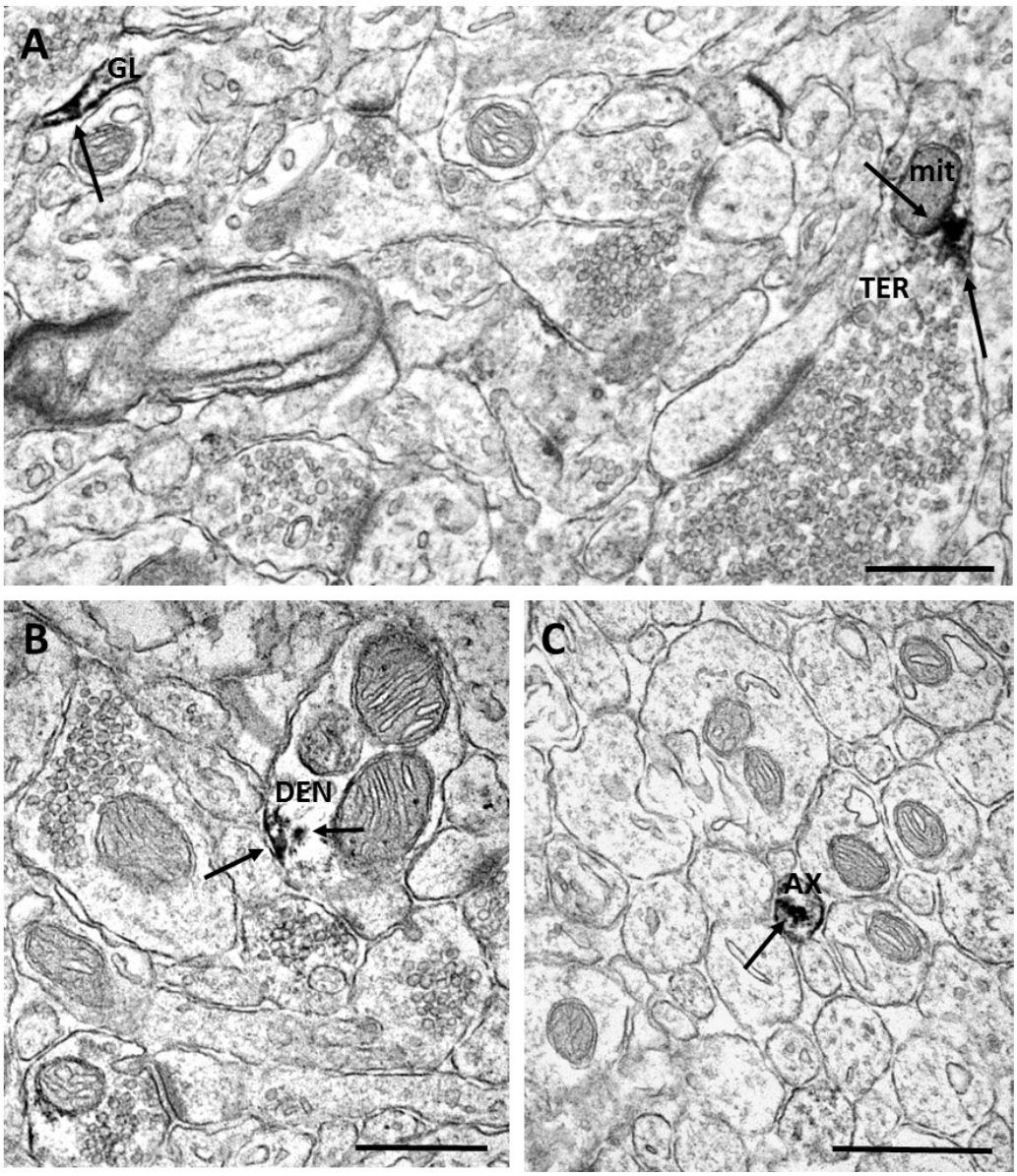

Figure 3. Electron micrographs show examples of ERβ-containing profiles in the NAc Core and Shell. **A)** ERβ-IR is observed in a terminal (TER), where it is localized to a mitochondrion (mit), small synaptic vesicles, and the plasma membrane. ERβ-IR is also associated with the membrane of a glial cell (GL) in the NAc Shell; **B)** the membrane and microtubules of a dendrite (DEN) in the NAc Core; **C)** an axon (AX). *Black arrow*, Immunoperoxidase for ER. *Scale bar*, 500 nm.

immunoreactivity in dendritic spines: 2% of GPER1-IR profiles in the NAc Core and 4% of GPER1-IR profiles in the NAc Shell (Figure 4C). In the dendritic shafts, GPER1 was typically associated with the plasma membrane, but also was affiliated with microtubules and mitochondrial membranes. In dendritic spines, GPER1 peroxidase reaction product accumulated in the spine head, and was associated with the plasma membrane, particularly near the post-synaptic density. Although GPER1 immunoreactivity was observed both pre and post-synaptically, it was rare for GPR1-IR terminals to synapse onto GPER1-IR spines. Immunoperoxidase for GPER1 was observed in neuronal perikarya, where it was commonly associated with organelles, including mitochondria and Golgi bodies (Figure 4B). Finally, 16% and 14% of GPER1-IR profiles were observed in glia in the NAc Core and NAc Shell, respectively; the labeling in glial cells was discrete, and was localized primarily to the plasma membrane (Figure 4A and B).

3.2 Experiment 2: Dual labeling for ERs and TH

In dual labelled sections, immunoreactivity for both ERα and GPER1 were observed in similar proportions to that seen in experiment 1. However, there was higher total labeling for GPER1 in this dual labeling study compared to the single labeling experiment (see Tables 1-3). In agreement with previous studies (Sesack & Pickel, 1990), immunogold labeling for TH was observed throughout the NAc Core and Shell in axons and axon terminals. TH immunoreactivity was most commonly observed in terminals that were 0.3-1.5µm diameter and contained numerous closely packed round small synaptic vesicles. TH-IR terminals sometimes formed symmetric synapses with dendrites and dendritic spines. TH immunolabelling was also infrequently observed in unmyelinated axons (0.1-0.2µm diameter).

There were low levels of co-localization between TH and both ERα and GPER1 in the NAc (See Tables 3). In the NAc Core, 1% of ERα-IR axons were also TH-IR, and 6% of ERα-IR axon terminals contained TH immunoreactivity. In the NAc Shell there were slightly higher levels of co-localization between ERα and TH: 7% of ERα-IR axons also contained TH immunoreactivity, and 11% of ERα containing axon terminals were TH-IR (Figure 5A and C). Co-localization of GPER1 and TH was also observed in the NAc Core and Shell. In the NAc Core, 7% of GPER1-IR axons also contained TH immunoreactivity and 12% of GPER1-IR axon terminals also contained TH immunoreactivity. In the NAc Shell 2.8% of axons labelled for

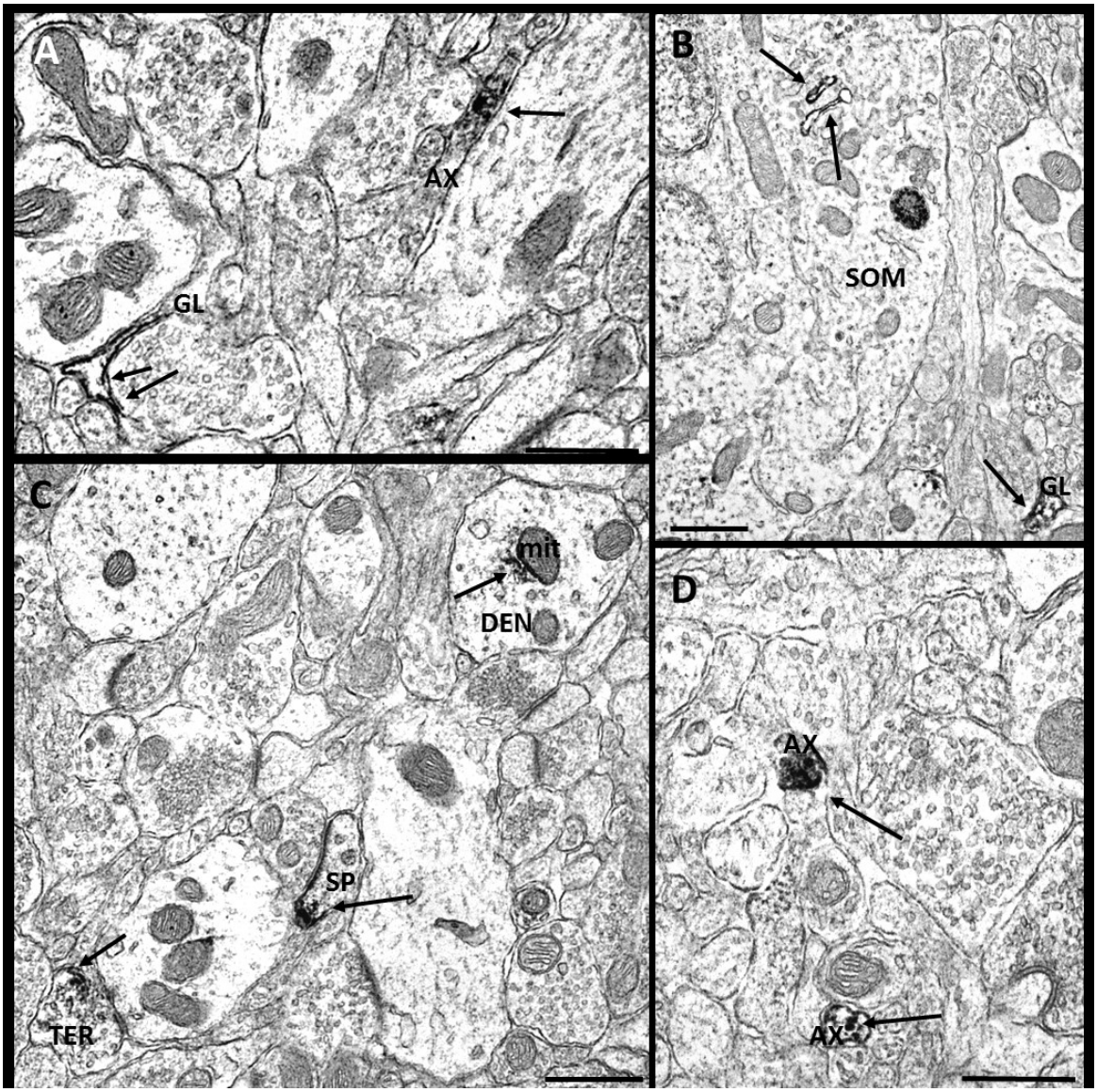

Figure 4. Electron micrographs show examples of GPER1-containing profiles in the NAc Core and Shell. GPER1immunoreactivity is observed in: **A)** an axon (AX) and associated with the membrane of a glial process (GL) in the NAc Shell; **B)** a soma where it is associated with Golgi bodies. GPER1immunoreactivity is also associated with the membrane of a glial cell (GL) in the NAc Core; **C)** a dendrite (DEN), where it is associated with the membrane of a mitochondrion (mit), a dendritic spine (SP) and vesicles in an axon terminal (TER), in the NAc Shell; **D)** in two axons in the NAc Core. *Black arrow*, Immunoperoxidase for ER. *Scale bar*, 500 nm.

GPER1 were also TH-IR, and 14.2% of all GPER1-IR axon terminals were TH-IR (Figure 5B and D). These findings demonstrate that a small proportion of ERα and GPER1 –IR profiles in the NAc are catecholaminergic neurons.

3.3 Experiment 3: Dual labeling for ERs and GABA

The proportions of both ERα and GPER1 observed in the NAc Core and Shell were comparable to those observed in the single label experiments, increasing confidence in these findings. Again, the total number of GPER1-IR profiles were higher in this experiment than in the single label experiment (see Table 1, 2 and 4), but were comparable to the total number of GPER1-IR profiles observed in the dual labeling experiment with TH (see Table 3 and 4). Immunogold labeling for GABA paralleled previous findings, with GABA immunoreactivity most commonly observed in terminals, dendrites, and perikarya of neurons in the NAc (Delle Donne et al., 1997; Van Bockstaele & Pickel, 1995). GABA immunoreactivity was also occasionally observed in axons and dendritic spines, but this was infrequent.

There are moderate levels of colocalization between ERα and GABA immunoreactivity in the NAc. Colocalization between ERα and GABA-IR was most commonly observed in dendritic shafts in both the NAc Core and Shell. In the NAc Core 53% of ERα-IR dendritic shafts were GABAergic, and in the NAc Shell 50% of ERα-IR dendrites also contained GABA immunoreactivity (see Table 4; Figure 6A). Colocalization between ERα and GABA was also frequently observed in axon terminals; 47% of ERα-IR terminals in the NAc Core and 37% of ERα-IR terminals in the NAc Shell also contained GABA-IR (Figure 6B). Lower levels of colocalization between ERα and GABA immunoreactivity were observed in axons and dendritic spines. Three percent of ERα-IR axons in the NAc Core, and 2% of ERα-IR axons in the NAc Shell were GABAergic. Furthermore, 5% of ERα-IR dendritic spines in the NAc Core and 8% of ERα-IR dendritic spines in the NAc Shell also contained GABA immunoreactivity. Glial profiles containing immunolabelling for ERα and GABA were also observed infrequently, with 3% and 5% of ERα-IR glia also containing GABA immunoreactivity in the NAc Core and Shell, respectively. Additionally, ERα was occasionally observed in soma containing GABA immunoreactivity, although these profiles were not included in quantification analyses.

Levels of colocalization between GPER1 and GABA in the NAc Core and Shell was also observed. GPER1 immunoreactivity was most frequently colocalized with GABA immunoreactivity in dendritic shafts; 52% of GPER1-IR dendrites in the NAc Core and 36% of

Table 3

ERα and GPER1 distribution in profiles containing TH in the Nucleus Accumbens

		ERα or GPER-1 + TH in Nac Core				ERα or GPER-1 + TH in NAc Shell			
Receptor		ERα	GPER-1	ERα + TH	GPER-1 + TH	ERα	GPER-1	ERα + TH	GPER-1 + TH
Dendrites	%	9.4	11.37	-	-	10.5	13.1	-	-
	# ±SEM	19.7 ±1.2	29.0 ±4.5	-	-	20.3 ±0.3	31.0 ±3.1	-	-
Spines	%	4.0	2.1	-	-	4.5	3.0	-	-
	# ±SEM	8.3 ± 0.8	5.3 ±1.2	-	-	8.7 ± 0.7	7.0 ±1.5	-	-
Axons	%	38.3	35.4	1.25	6.6	38.1	40.4	7.2	2.8
	# ±SEM	80.0 ±9.7	90.3 ±2.9	1.0 ±0.8	6.0 ±1.6	74.0 ±9.3	95.7 ±5.8	5.3 ±0.7	2.7 ±1.2
Terminals	%	33.2	29.9	6.3	12.2	28.5	31.8	11.4	14.2
	# ±SEM	69.3 ±1.2	76.3 ±7.8	4.3 ±0.4	9.3 ±2.4	55.3 ±3.4	75.3 ±11.7	6.3 ±1.3	10.7 ±5.3
Glia	%	15.2	10.2	-	-	18.5	10.7	-	-
	# ±SEM	31.7 ±5.2	26.0 ±5.0	-	-	36.0 ±2.0	25.3 ±1.2	-	-
Total	%	100.0	100.0	2.6	6.5	100.0	100.0	6.0	5.7
	# ±SEM	209.0 ±15.0	255.0 ±7.6	5.3 ±0.4	16.7 ±4.9	194.3 ±9.7	236.7 ±13.5	11.7 ±1.6	13.7 ±6.9

The percentage of total IR profiles the number of IR profiles, and the corresponding standard error, observed in ~6000µm area of the NAc Core and Shell, averaged across rats.

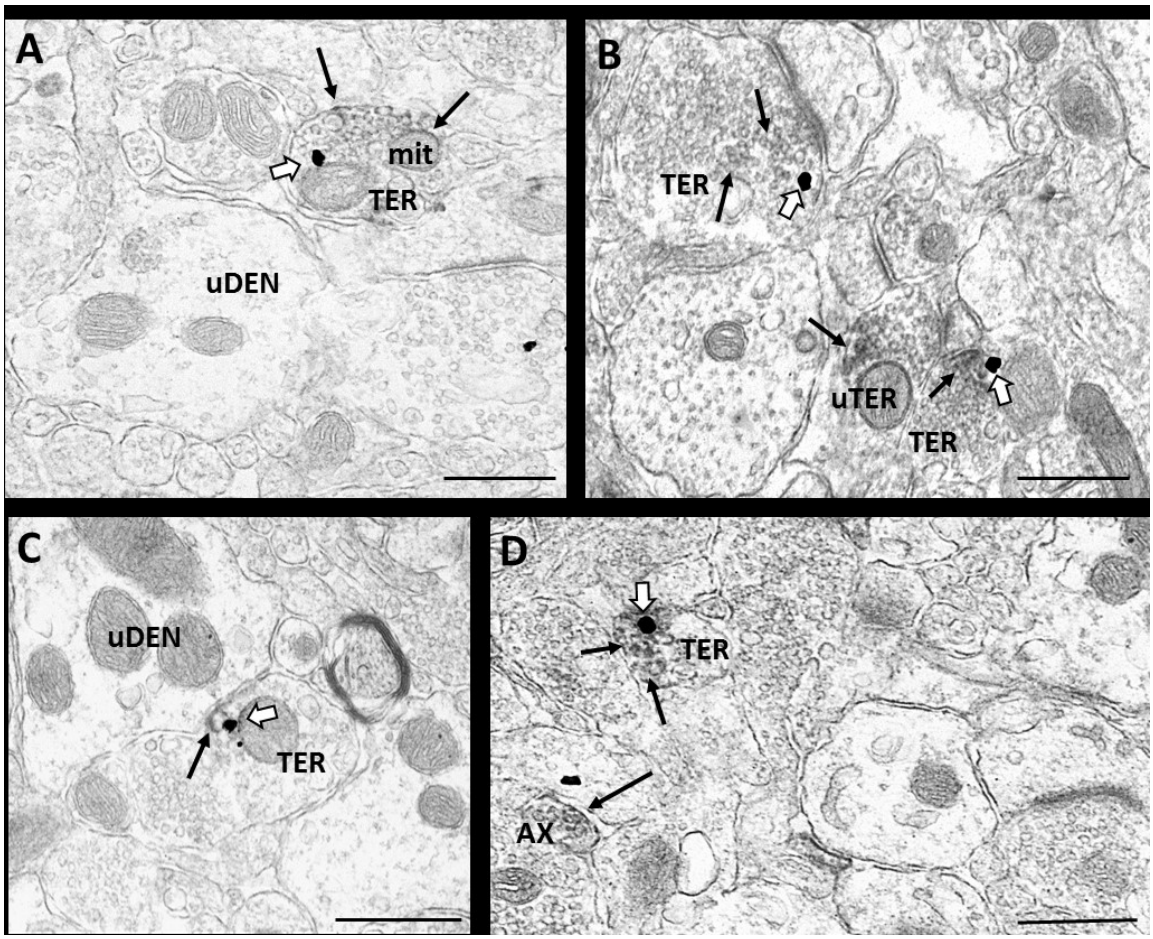

Figure 5. Electron micrographs show examples of profiles containing of ERα or GPER1-immunoreactivity and Tyrosine Hydroxylase (TH) immunoreactivity in the NAc Core and Shell. **A)** Immunoreactivity for ERα is associated with small synaptic vesicles and a mitochondrion in a catacholeminergic terminal (TER) that is adjacent to an unlabeled dendrite (uDEN). **B)** GPER1 immunoreactivity associated with synaptic vesicles close to the synapse in two catecholeminergic terminals (TER), and one non-catecholaminergic terminal (uTER). **C)** ERα-IR catecholaminergic terminal (TER) in close proximity to a synapse with an unlabeled dendrite (uDEN). **D)** GPER1 immunoreactivity associated with synaptic vesicles in a catecholaminergic terminal (TER), and an axon (AX). *Black arrow*, Immunoperoxidase for ER; *white arrow*, immunogold for TH. *Scale bar*, 500 nm.

Table 4

ERα and GPER1 distribution in profiles containing GABA in the Nucleus Accumbens.

		ERα or GPER-1 + GABA in NAc Core				ERα or GPER-1 + GABA in NAc Shell			
Receptor		ERα	GPER-1	ERα + GABA	GPER-1 + GABA	ERα	GPER-1	ERα + GABA	GPER-1 + GABA
Dendrites	%	4.8	4.4	53.3	51.8	4.7	4.4	50.0	35.6
	# ±SEM	10.0 ±2.0	28.3 ±3.2	5.3 ±1.8	14.6 ±1.2	9.3 ±0.9	29.0 ±2.5	4.6 ±0.4	10.3 ±0.7
Spines	%	3.3	4.0	4.8	13.6	4.2	4.0	8.0	8.7
	# ±SEM	7.0 ± 1.5	7.3 ±1.2	0.3 ±0.3	1.0 ±0.8	8.3 ± 0.3	7.7 ±0.7	0.7 ±0.8	0.7 ±0.7
Axons	%	37.5	36.8	3.4	3.2	39.1	36.8	1.7	1.3
	# ±SEM	79.0 ±2.9	94.0 ±1.5	2.7 ±1.7	3.0 ±1.6	77.3 ±3.2	75.7 ±2.3	1.3 ±0.4	1. ±0
Terminals	%	43.1	43.7	46.7	40.7	40.8	43.7	37.2	33.8
	# ±SEM	90.7 ±3.2	84.3 ±2.3	42.3 ±4.4	34.3 ±4.5	80.7 ±5.8	76.0 ±10.6	30.0 ±6.5	25.7 ±3.2
Glia	%	11.6	11.1	2.7	5.0	11.1	11.1	4.5	2.8
	# ±SEM	24.3 ±3.2	20.0 ±2.1	0.7 ±0.7	1.0 ±0.4	22.0 ±1.5	23.7 ±3.4	1.0 ±1.2	0.7 ±0.3
Total	%	100.0	100.0	24.4	23.3	100.0	100.0	19.1	18.1
	# ±SEM	210.3 ±5.6	237.0 ±5.2	51.3 ±2.2	55.3 ±0.8	197.6 ±7.3	212.0 ±7.0	37.7 ±4.1	38.3 ±3.5

The percentage of total IR profiles, the number of IR profiles, and the corresponding standard error observed in ~6000µm area of the NAc Core and Shell, averaged across rats.

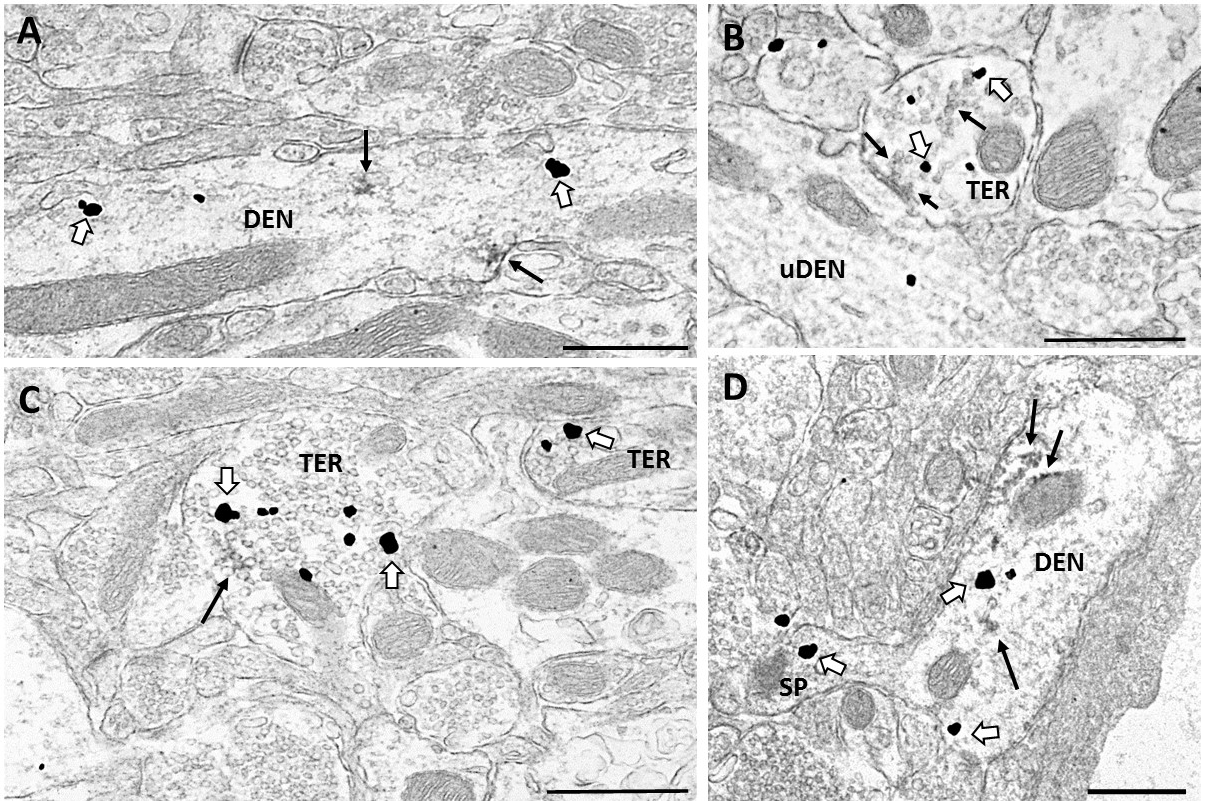

Figure 6. Electron micrographs show examples of profiles containing of ERα or GPER1-IR and GABA-IR in the NAc Core and Shell. A) ERα-IR is associated with microtubules and the plasma membrane of a GABAergic dendrite (DEN). B) ERα-IR associated with synaptic vesicles and the membrane near a synapse in a GABAergic terminal (TER). C) GPER1-IR associated with synaptic vesicles in a GABAergic terminal (TER); D) a GABAergic dendrite (DEN) with a spine (SP). *Black arrow*, Immunoperoxidase for ER; *white arrow*, immunogold for GABA. *Scale bar*, 500 nm.

GPER1-IR dendrites in the NAc Shell were GABAergic (see Table 4; Figure 6D). Colocalization was also observed in axon terminals, as 41% of GPER1-IR axon terminals in the NAc Core, and 34% of GPER1-IR terminals in the NAc Shell also contained GABA immunoreactivity (Figure 6C). Lower levels of colocalization were observed in spines with 13% of GPER1-IR dendritic spines in the NAc Core and 9% of the spines in the NAc Shell also containing GABA immunoreactivity. Low levels of colocalization were observed between GABA and GPER1 in axons, with 3% of GPER1-IR axons in the NAc Core and only 1% of GPER1-IR axons in the NAc Shell, also containing GABA immunoreactivity. Finally, low levels of colocalization of GPER1 and GABA were also observed in glia in the NAc; 5% of GPER1-IR glia in the NAc Core and 3% of GPER1-IR glia in the NAc Shell also contained GABA immunoreactivity. GPER1 immunoreactivity was observed in GABAergic soma, but again these were not included in quantification analyses.

4. Discussion

Ultrastructural analysis demonstrates that ERα, ERβ, and GPER1 are localized to extranuclear sites in the NAc Core and Shell of female rats. Although these receptors are observed at all types of neuronal profiles and in glia, the majority are observed at presynaptic sites. The distribution of the ERs in the NAc Core and Shell is very similar, indicating that ERs do not differ between these subregions of the NAc. Additionally, these experiments indicate that a very low proportion of ERα and GPER1 are localized to catecholaminergic neurons, and a moderate proportion of ERα and GPER1 are localized to GABAergic neurons in the NAc.

4.1 Methodological Considerations

Methodological considerations are discussed in detail in previous publications (Almey et al., 2012; Milner et al., 2001). Briefly, the immunolabelling methods used here lead to excellent preservation of cellular morphology allowing for discrete localization of antigens (Leranth C., 1989). All tissue sections were identical in size and were taken near the plastic tissue interface to prevent differences in antigen penetration from affecting the results of these experiments (Milner et al., 2011). Immunoreactivity for ERα, ERβ, and GPER1 is discrete, and a very thin plane of section is examined, so a lack of ER immunoreactivity does not demonstrate that these profiles lack ERs. For the same reason, the probability of detecting both peroxidase and gold immunomarkers in the same plane of section, particularly for small profiles, is decreased. As a

result, the semiquantitative analyses presented here are likely conservative, underestimating the number of ERs and the frequency with which these receptors are localized to profiles containing TH or GABA.

4.2 ERα is observed at extranuclear sites

ERα was the most frequently observed ER in the NAc, and results of the single and dual labeling experiments were similar, observing comparable levels of ERα-IR. Post-synaptic labeling was slightly lower in the NAc Shell than Core, but in general, these analyses indicate that ERα distribution does not differ between subregions of the NAc. ERα was observed in all types of neuronal profiles and in glial cells in both the NAc Core and Shell, differing from previous light microscopy experiments that observe relatively low levels of nuclear labeling for ERα in the NAc (Mitra et al., 2003; Shughrue et al., 1998). This discrepancy is likely due to the increased resolution of electron microscopy, allowing for detection of the mERα-IR profiles observed in this experiment.

Although ERα-IR was observed in all types of neuronal profiles, ERα was most commonly localized to presynaptic profiles, axons and axon terminals. The presence of ERα in axons may simply reflect the transportation of this receptor to terminals, but these binding at receptors on axons can affect the transmission of action potentials and protein transport (Carr, Sittl, Fleckenstein, & Grafe, 2010; Verdier, Lund, & Kolta, 2003). ERα in axon terminals is positioned to affect the transportation of vesicles to the synapse, which has been observed in hippocampal neurons (Hart, Snyder, Smejkalova, & Woolley, 2007). ERα in terminals is also positioned to affect the release of transmitter from terminal, providing a mechanism for the finding that E2 increases phasic dopamine release in the NAc (Thompson & Moss, 1994,1997). ERα was also localized to dendrites and dendritic spines, but was not frequently observed in these post-synaptic profiles. In addition to being localized to neurons, 10% of ERα is also observed at the membrane of glial cells. Estrogen is involved in glial mediated neuroprotection (Arevalo, Santos-Galindo, Bellini, Azcoitia, & Garcia-Segura, 2010;Spence & Voskuhl, 2012), and these membrane associated ERs provide a mechanism for this effect. These experiments observed ERα primarily at extranuclear sites, which corresponds to previous findings in the STR (Almey et al., 2012).

4.3 ERβ is observed at extranuclear sites

These analyses observed ERβ in all types of neuronal profiles and in glial cells in both the NAc Core and Shell. ERβ was observed approximately half as frequently as ERα in the NAc, suggesting that binding at ERβ would have less effect on transmission in the NAc, although experiments with ERα and ERβ agonists are needed to confirm this. Post-synaptic labeling for ERβ was slightly lower in the NAc Core than Shell, but generally these analyses indicate that ERβ distribution does not differ between subregions of the NAc. These experiments observe moderate levels of extranuclear ERβ-IR in the NAc, which differs from previous light microscopy findings showing only low levels of nuclear labeling for ERβ in this region (Mitra et al., 2003; Shughrue et al., 1998). Again, this discrepancy is likely due to the increased resolution of electron microscopy.

ERβ was also primarily observed presynaptically, in axons and axon terminals. Similar to ERα, ERβ in axons could affect the transmission of action potentials and protein transport (Carr et al., 2010; Verdier et al., 2003), and ERβ in axon terminals is positioned to affect the release of transmitter from terminal in the NAc. ERβ is also observed at postsynaptic profiles, including dendrites and dendritic spines, albeit at much lower levels. There were also glia in the NAc containing ERβ-IR, indicating that binding at these receptors could also contribute to estrogens' effects on glial-mediated neurotransmission (Arevalo et al., 2010). These experiments observed ERβ almost exclusively at extranuclear sites in the NAc, which corresponds to previous findings that estrogens rapidly alter transmission in the NAc (Le Saux et al., 2006; Thompson & Moss, 1994).

4.4 GPER1 is observed at extranuclear sites

GPER1 was also observed at all types of neuronal profiles in the NAc Core and Shell. To the best of our knowledge, this is the first paper to report that GPER1 is prevalent in the NAc, as previous light microscopy experiments observe relatively low levels of GPER1 in this region (Hazell et al., 2009). These receptors were observed at extranuclear sites using light microscopy, which was confirmed by ultrastructural analysis. The semiquantitative analyses of the single and dual labeling studies indicate that the distribution of GPER1 does not differ between subregions of the NAc. However, there was a discrepancy between single and dual labeling studies; these studies observe similar proportions of GPER1 immunoreactivity in the various types of neuronal processes, but the single labeling experiment observed ~25% less GPER1-IR profiles than the

two dual labeling studies. There are a number of potential causes for this discrepancy. It could result from the fact that sections in the single labeling study may have been from slightly deeper in the tissue section reducing antibody permeability. Alternately, a difference in counterstaining of tissue could be responsible for the difference, resulting in darker background in the single labeling study and greater difficulty observing peroxidase reaction product. Since the total number of profiles observed is very similar between the two dual labeling studies, it is hypothesized that these studies are the most accurate representation of total GPER1 in the NAc, suggesting the GPER1 and ERα have similar prevalence in this region.

Like ERα and ERβ, GPER1 is observed primarily in presynaptic profiles in both the NAc Core and Shell. GPER1 in axons and axon terminals is positioned to affect the propagation of action potentials, protein transportation and the release of neurotransmitters from terminals (Carr et al., 2010; Thompson & Moss, 1994; Verdier et al., 2003). GPER1-IR was also observed in post-synaptic profiles in the NAc, including dendritic shafts and spines, but this occurred much less frequently. Glia in the NAc Core and Shell were IR for GPER1, suggesting that estrogens effects on glial-mediated neuroprotection could also occur through binding at this receptor (Arevalo et al., 2010; Spence & Voskuhl, 2012).

These single labeling experiments clearly demonstrate that ERα, ERβ, and GPER1 are localized to extranuclear neuronal sites in the NAc, and that levels of these receptors do not differ between the Core and Shell subregions of the NAc. It is postulated that there are similar levels of ERα and GPER1 in the NAc, while ERβ is less prevalent, occurring half as frequently as the other ERs. As discussed above, all three ERs were observed in glia, which could explain how estrogens contribute to glial-mediated neuroprotection. Additionally, similar to findings in the STR and hippocampus, ERα, ERβ, and GPER1 are commonly associated with the membranes of mitochondria in dendrites, terminals, and soma. Binding at ERs associated with mitochondria could contribute to estrogens effects on cellular metabolism observed in neurons and glia (Araujo, Beyer, & Arnold, 2008; Razmara et al., 2008). E2 infused into the NAc rapidly increases phasic dopamine release (Thompson & Moss, 1994) and increases D2 receptor binding without affecting D2 mRNA, suggesting that these changes in D2 receptor density occur via binding at membrane-associated receptors (Le Saux et al., 2006). Binding at the membrane associated ERα, ERβ, or GPER1 observed in these experiments could cause these rapid E2-induced changes in transmission in the NAc.

4.5 Low levels of ERα and GPER1 are localized to catecholaminergic neurons in the NAc

The dual labeling analyses used in these experiments demonstrated that a low proportion of ERα-IR and GPER1-IR were localized to profiles containing TH. ERα was colocalized with TH more frequently in the NAc Shell (~7% of all ER-IR profiles) than in the NAc Core (~3% of profiles), but there was no difference in GPER1 colocalization with TH in the Core and Shell (~7% of profiles). The highest level of colocalization between both ERα and GPER1 and TH was observed in catecholaminergic terminals; if these neurons are in fact dopaminergic, binding here could account for the rapid effects of estrogen on presynaptic dopamine transmission in the NAc (Thompson & Moss, 1994, 1997).

4.6 Moderate levels of ERα and GPER1 are localized to GABAergic neurons in the NAc

These analyses demonstrated that a relatively large proportion of ERα and GPER1 were localized to GABAergic neurons in the NAc Core and Shell. There were similar levels of ERα-GABA colocalization and GPER1-GABA colocalization in the NAc Core and Shell, although colocalization of ERα-IR and GABA-IR was slightly lower in the NAc Shell than in the NAc Core. The greatest proportion of ERα and GPER1 colocalization with GABA was observed in dendritic shafts, as ~50% of ERα and GPER1 –IR dendrites were GABAergic. A substantial proportion colocalization was observed in axon terminals, as ~40% of ERα and GPER1 –IR terminals were GABAergic. Low levels of colocalization between ERα/GPER1 and GABA were observed in axons and dendritic spines, likely due, in part, to the low levels of GABA immunoreactivity observed in these profiles (Pickel, Towle, Joh, and Chan, 1988). Finally, ERα and GPER1 were infrequently localized to GABA-IR glial cells. These findings of clearly demonstrate that some of the ERα and GPER1 in the NAc are localized to GABAergic neurons and glia. This parallels findings in the STR, which observed ERα and GPER1 in GABAergic dendrites and terminals (Almey, Milner and Brake, Chapter 4B). Taken together, this previous study and the current experiments suggests similarities in the distribution of mERs in the STR and the NAc (aka the ventral striatum).

Little research has examined the effect of estrogens on GABA in the NAc, but the presence of ERα and GPER1 in GABAergic terminals suggests that estrogens could alter GABA availability. Changes in GABA transmission in the NAc affect dopamine availability; decreasing GABA transmission by antagonizing $GABA_A$ receptors increases phasic dopamine release (Xi and Stein, 1998), and modafinil and neurotensin –induced changes in dopamine release in the

NAc are mediated by GABA (Ferraro, Tanganelli, O'Connor, Antonelli, Rambert and Fuxe, 1996; Tanganelli, O'Connor, Ferraro, Bianchi, Beani, Understedt and Fuxe, 1994). In the STR estrogens rapidly (<1hour) decreases extracellular GABA (Hu et al., 2006; Schultz et al., 2009); the distribution of ERα and GPER1 are similar in the STR and NAc, so research should examine whether estrogens also rapidly decrease GABA availability in the NAc. Together, these findings suggest that E2 may indirectly increase dopamine availability in the NAc by reducing GABA transmission. Additionally, TH terminals often synapse onto dendrites of spiny interneurons in the NAc, which are presumed to be GABAergic (Sesack & Pickel, 1990). The ERα and GPER1 observed in GABAergic dendrites in these experiments are ideally positioned to alter transmission at these synapses. This provides a second mechanism through which estrogens could alter catecholaminergic transmission in the NAc.

CHAPTER 6:
MEDIAL PREFRONTAL CORTICAL ESTRADIOL RAPIDLY ALTERS MEMORY SYSTEM BIAS IN FEMALE RATS: ULTRASTRUCTURAL ANALYSIS REVEALS MEMBRANE-ASSOCIATED ESTROGEN RECEPTORS AS POTENTIAL MEDIATORS.

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Preface:

Following the success of the electron microscopy experiments in the STR and NAc it was important to extend the findings to the PFC, examining the distribution of ERs in that region. In addition, it was important to determine whether binding at membrane associated receptors rapidly altered behaviour, yet little research has addressed this. To provide an unequivocal demonstration that estrogens have rapid effects on neurotransmission to induce rapid behavioural changes, 17β-estradiol (E2) was infused directly into the PFC, instead of using systemic administration. Additionally, a behavioural test that could be completed within 20 minutes of the E2 infusion was chosen; E2 could not alter production of protein this rapidy since the most rapid changes in protein production (expression of immediate early genes) take at least 30 minutes to occur (Sokolova, Shtark, Lisachev, Pustyl'nyak, and Pan, 2009). Based on this, we decided to assess the effects of E2 infusions in the PFC on multiple memory system bias.

Multiple memory system bias was chosen over other cognitive processes examined in this thesis for two reasons. First, tests for perseveration and reversal learning take at least 1 hour, often longer in the case of reversal learning, which is tested across two days. Thus, there could be some ambiguity as to whether any effects of E2 infusion observed in the test results were caused by long-term or rapid effects. The LI test (Chapter 2) takes under 30 minutes, but for E2 to affect the expression of LI it must be administered prior to the conditioning session, not the test session (Nofrey, Ben Shahar and Brake, 2007). Additionally, contradictory results surrounding the effects of E2 on LI indicate that this behaviour might be particularly sensitive to the timing/dose of E2 administered (Arad and Winer, 2010a and 2010b; Nofrey, Ben Shahar and Brake, 2007; Quinlan et al., 2010). Multiple memory systems are consistently altered by E2 administration, with elevated plasma levels of E2 associated with a bias towards place memory (Korol, 2004; Korol et al. 2004; Korol and Kolo, 2002; Quinlan et al., 2008; 2013; 2014). Also, the structure of this task is such that the ability to perform to criterion can be assessed prior to the probe trial to ensure that the infusion of E2 to the PFC did not affect the ability to navigate the maze, just navigation strategy.

Similar to other cognitive processes examined in this thesis, multiple memory system bias is also affected in schizophrenic individuals. Specifically, individuals with schizophrenia exhibit deficits in spatial navigation, but intact egocentric navigation (Spieker, Astur, West,

Griego and Rowland, 2012; Weniger and Irle, 2008), which correspond to abnormal hippocampal and prefrontal cortical activation (Baare, van Oel, Hulshoff, Schnack, Durston, Sitskoorn and Kahn, 2001; Ledoux, Phillips, Labelle, Smith, Bohbot and Boyer, 2013). However, for this experiment we did not examined multiple memory systems in the context of an animal model of schizophrenia, nor did we administer any HAL to assess the effects of HAL administered alone and in conjunction with E2 on navigational strategies. Although these experiments would also be very interesting, an initial experiment to determine whether E2 in the PFC had any effect on multiple memory system bias was required before introducing additional pharmacological manipulations.

Abstract

High plasma levels of estradiol are associated with use of a place memory system over a response memory system. We examined whether infusing E2 into the medial prefrontal cortex (mPFC) or anterior cingulate cortex (AC) could affect memory system bias in female rats. We also examined the ultrastructural distribution of ERs ERα, ERβ, and GPER1 in the mPFC of female rats as a mechanism for the behavioural effects of E2 in the mPFC. Each rat was infused bilaterally with either E2 (0.13μg) or vehicle into the mPFC or AC. The majority of E2 mPFC rats used place memory. In contrast, the majority of mPFC vehicle rats and AC E2 or vehicle rats used response memory. These data show that mPFC E2 rapidly biases females to use place memory. Electron microscopic analysis demonstrated that ERα, ERβ, and GPER1 are localized in the mPFC, almost exclusively at extranuclear sites. This is the first time that GPER1 has been localized to the mPFC of rats, and the first time that ERα and ERβ have been described at extranuclear sites in the rat mPFC. The majority of receptors were observed on axons and axon terminals, suggesting that estrogens alter presynaptic transmission in the mPFC. This provides a mechanism via which ERs could rapidly alter transmission in the mPFC to alter PFC dependent behaviours, such as memory system bias. The discrete nature of immunolabelling for these membrane-associated ERs may explain the discrepancy in previous light microscopy studies.

1. Introduction:

Several strategies can be used when solving a maze to obtain a reward. One is response memory, which involves specific motor responses required to obtain a reward (Tolman, Ritchie, & Kalish, 1946), meaning a rat will learn to always turn left or right in a maze. Alternately, place memory refers to the use of distal cues around the maze to orient to a location; these cues are compiled into a cognitive map that is used to navigate in the maze (Tolman et al., 1946). White and McDonald (White & McDonald, 2002) proposed that that response and place memory strategies are supported by two independently functioning parallel memory systems. The hippocampal system is thought to process information about environmental cues, as is done when using place memory. The second memory system is the dorsal striatal system, which is believed to support stimulus-response learning; in this form of learning an animal performs a habitual response when presented with a stimulus, as is done when using response memory. In some cases the effectiveness of one system can be increased by disabling the other (Packard, Hirsh, and White, 1989).

In females these memory systems are sensitive to fluctuations in levels of estrogens (for review see (Hussain, Hoehne, Woodside, & Brake, 2013; Korol, 2004)). When estrogen levels are high, female rats are biased towards use of place memory (Korol & Kolo, 2002; Korol, Malin, Borden, Busby, & Couper-Leo, 2004; Quinlan et al., 2013; Quinlan et al., 2008). In contrast, when estrogen levels are low female rats are biased towards use of response memory (Korol & Kolo, 2002; Korol et al., 2004; Quinlan et al., 2013; Quinlan et al., 2008). Injection of E2 into the dorsal hippocampus has been found to improve place learning, while injections of E2 into the STR impair response learning (Zurkovsky, Serio, & Korol, 2011). These results suggest increases in systemic estrogens bias females towards use of place memory, due to estrogens acting in the hippocampus. Lower levels of estrogens are associated with a bias towards use of response memory.

The hippocampus and the STR have reciprocal projections to the mPFC, so it is possible that this area may influence the outputs of these two systems (White & McDonald, 2002). Neurons in the prelimbic and infralimbic regions of the mPFC are activated in response to a switch from place to response memory, but not in response to changes in behavioural or task contingencies (Rich & Shapiro, 2009). These findings suggest that the mPFC plays a role in determining whether place or response memory will be used. Additionally, estrogens may

contribute to mPFC control of memory system bias; OVX female rats with low E2 replacement switch from a response to place memory when dopamine D1 or D2 receptor antagonists are infused into the mPFC, while females administered high doses of E2 used a place memory regardless of dopamine antagonist administration (Quinlan, Radiotis, Lachapelle, Caisse, & Brake, 2014). Such results support a role for the mPFC in the E2-induced bias towards use of place memory.

The mechanisms by which estrogens could alter neurotransmission in the mPFC to elicit these effects on multiple memory systems remain unclear. Estrogens act by binding at both nuclear and membrane-associated ERs to elicit rapid changes in cell firing and slower alterations in protein production. In terms of the classical ERs, previous research has yielded contradictory results on the distribution of these receptors in the mPFC. Most studies demonstrate little ERα immunoreactivity (IR) in the mPFC of rats (Cardona-Gomez, DonCarlos, & Garcia-Segura, 2000; Kritzer, 2002), and low levels of ERβ immunoreactivity (Kritzer, 2002) and mRNA (Shughrue & Merchenthaler, 2001) in the mPFC of female rats. However, other studies demonstrate moderate levels of ERα-IR in the mPFC of rats (Montague et al., 2008; Wang, Hara, Janssen, Rapp, & Morrison, 2010), and moderate levels of ERβ mRNA in the mPFC of mice (Mitra et al., 2003). These studies observe these ERs exclusively at nuclear sites in the mPFC. One possible reason for the discrepancy in previous findings is that light microscopy, unlike electron microscopy (EM), is not sensitive enough to detect ERs at cell membranes in the mPFC. This assertion is supported by the finding that ultrastructural analysis detects abundant ERα immunoreactivity at the cell membrane in the primate PFC (Wang et al., 2010), which is not observed with light microscopy in rodents (Cardona-Gomez et al., 2000; Kritzer, 2002; Montague et al., 2008; Shughrue & Merchenthaler, 2001; Wang et al., 2010). However, further research is needed to clarify whether ERα and ERβ are localized to the extranuclear sites in the mPFC of female rats, and to investigate if the most recently-discovered ER, GPER1, is also present in the rat mPFC.

This experiment was conducted to determine whether estrogens act in the mPFC to bias female rats towards use of place memory when solving an appetitive task. Here OVX female rats administered chronic low E2 replacement were implanted with bilateral cannulae in the mPFC or the anterior cingulate cortex (AC). The AC was selected as a control brain region because it is immediately dorsal to the mPFC, so any effect of E2 could be attributed to the mPFC, and not E2

diffusion to dorsal regions along the cannula tract. All rats received microinfusions of E2 and vehicle, in counterbalanced order, immediately prior to navigating a maze task that could be completed using either place or response memory. Additionally, tissue from the mPFC of female rats in the diestrus phase of the cycle was immunolabelled for ERα, ERβ, and GPER1, and was examined using EM.

2. Method

2.2 Experiment 1 – mPFC and memory systems bias

Animals. This experiment used 32 female Sprague-Dawley rats (Charles River, St. Constant, QC. Canada) that weighed 240-260g on arrival. Rats were pair-housed until surgery, after which they were individually housed. The colony room was maintained on a reverse 12hour light cycle, with lights off at 9:00 am. Standard lab chow and water were available ad libitum until the start of the experiment when food restriction began. The procedures used in the experiment were approved by the Concordia University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Surgery and hormone administration. Surgeries were conducted as described previously (Quinlan et al., 2013; Quinlan, Radiotis, Lachapelle, Caisse, & Brake, 2014). Cannulae were implanted (Plastics One, Roanoke, VA, USA) for microinfusion of E2 or cyclodextrin vehicle. For the mPFC group, coordinates from bregma were: AP = +3.1mm, ML = ± 1.5mm at 15º, and DV= –3.0mm from skull surface (Paxinos & Watson, 1998). For the AC group, the stereotaxic coordinates were: AP = +3.1, ML = ±1.35 at 15º, and DV = -1.5. During the same procedure, rats were OVX via a single lumbar incision, and implanted subcutaneously at the nape of the neck with a silastic capsule containing 5% E2 (Sigma Chemical Co., St. Louis, MO, USA) in cholesterol (Sigma). These implants have been shown to produce low E2 plasma levels, similar to levels observed during the diestrus phase of the estrous cycle (Almey et al., 2012). This low plasma level of E2 has previously been associated with a response memory bias (M. G. Quinlan et al., 2013; M. G. Quinlan et al., 2008). Following the procedure, rats were allowed one week to recover before training began.

E2 encapsulated in cyclodextrin and the cyclodextrin vehicle were dissolved in artificial cerebrospinal fluid immediately before the testing session began. Drugs were infused bilaterally using injectors that extended 1 mm beyond the end of the cannulae. Infusions were one minute,

at a rate of 0.5 μL/min, after which the injectors were left in place for another minute while the drug diffused. This dose of E2 has been shown to have behavioral effects when infused into the brain (Zurkovsky et al., 2007).

Apparatus, modified plus maze. Training was conducted using a modified plus maze, as previously described (Hussain et al., 2013; Quinlan et al., 2013; Quinlan et al., 2008; Quinlan, Radiotis, G., Lachapelle, I., Caisse, M., & Brake, W.G., 2014). During training trials, access to the probe start arm was blocked off, resulting in a T-shaped maze (Fig 1A), and prior to testing the experimenter unblocked the probe arm and blocked the start arm, creating a T-maze 180^{o} in orientation relative to the training T-maze (Fig 1B). At the end of each goal arm there was a bowl for the food reward (Kellogg's Froot Loops®), and Froot Loops crumbs were placed under the arms to mask any odor cues. There were extra-maze cues around the room to facilitate navigation the maze, and testing took place under illumination from 20W lights above each goal arm.

Procedure. The training and testing phases of the experiment have been described extensively elsewhere (Quinlan et al., 2013; Quinlan et al., 2008). Briefly, rats were food restricted and maintained at 90% of their free-feeding weight, and trained to find a Fruit Loops that was consistently located in one of two goal arms. Each rat received 10 training trials daily; during each trial the rat was placed in the start arm and permitted to enter either of the goal arms. The inter-trial interval was 10-60sec. Rats were trained daily until reaching criterion, which was eight out of ten correct trials for three consecutive days.

The day after the rats attained criterion, either E2 encapsulated in cyclodextrin (5.44 μg/mL: 5% 17β-E2, 95% cyclodextrin) or cyclodextrin vehicle (5.16 μg/mL) was infused bilaterally into the mPFC or AC. All rats were tested under both treatment conditions (E2 and vehicle), and order effects were controlled by counterbalancing. Immediately following the infusion, each rat underwent 10 trials; rats only underwent testing if they remained at criterion for these trials. After the 10th trial, the maze was inverted for testing. There were10-20 minutes between the infusion and the probe trial. During the probe trial, if the rat entered the goal arm that had been baited during the training phase they were considered to be using place memory. In contrast, if the rat entered the opposite goal arm, thereby making the same directional turn as during training, the rat was considered to be using response memory. Following the first probe

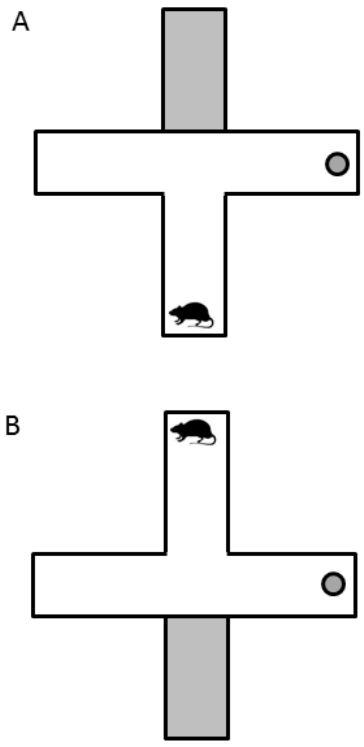

Figure 1. **A)** Maze orientation during training trials **B)** Maze orientation during probe trial used to determine what memory system is being used to navigate the maze.

trial, rats were retrained until they reached criterion, which took a minimum of 3 days of training. Then they were infused again with either E2 or vehicle, whichever one was not administered prior to the first probe trial, and underwent a second probe trial.

Histology. Following behavioral testing, rats were infused with methylene blue to mark cannula placements, and then they were decapitated. Their brains were removed, flash frozen in isopentane, and stored at -80ºC. Brains were sliced coronally on a cryostat at 40μm, and mounted on slides for confirmation of placements.

Statistical Analysis. This experiment is a within subjects experimental design with treatment (E2 or vehicle) as the within factor. Since the dependent variable is categorical, non-parametric statistical techniques were used to determine if there was a significant difference in memory system use under E2 and vehicle treatments. A McNemar test was used to compare the proportion of rats using each strategy following infusions of the two compounds in the mPFC and AC groups. An odds ratio and a logit *d* were computed to provide an estimate of effect size for each McNemar analysis (Klein, 2004).

2.2 Experiment 2 – Ultrastructural analysis of ERs in the mPFC

Animals. Six adult female Sprague Dawley rats from Charles River Laboratories (Wilmington, MA), approximately 225-250g on arrival, were pair-housed with *ad libitum* access to food and water and with 12:12 light/dark cycles, with lights on at 6:00am. Tissue from rats in the diestrus phase of the estrous cycle was analyzed for these experiments. Rats in the diestrus phase were used because this phase of the cycle corresponds to the low E2 replacement administered in the behavioural experiment. All procedures were in accordance with the National Institutes of Health guidelines and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. The rats used in these experiments are the same as those used by Williams et al. (Williams et al., 2011) and Almey et al. (Almey et al., 2012).

Antisera. For ERα identification, a rabbit polyclonal antiserum (AS409) produced against the full peptide for the native rat ERα was supplied by S. Hayashi. The specificity of this antibody has previously been demonstrated by binding to 3H-E2, immunoblots, and preadsorption controls (Alves et al., 1998; T. A. Milner et al., 2001; Okamura et al., 1992). For localization of ERβ, a rabbit polyclonal antiserum produced against a peptide sequence in the C-terminus of ERβ the mouse was used (Z8P; Zymed Laboratories, San Francisco, CA; (Shughrue & Merchenthaler, 2001). This antibody has been shown to be specific for ERβ by Western blot

analysis (~60 kDa), double label with mRNA using in situ hybridization, preadsorption control and absence of labeling in fixed brain sections from ERβ knockout mice (Creutz & Kritzer, 2002; Shughrue & Merchenthaler, 2001). Moreover, ERβ immunoreactivity colabels with green fluorescent protein in Esr2 transgenic mice (T. A. Milner et al., 2010). Finally, to visualize GPER1 this experiment used a rabbit polyclonal antiserum generated against a synthetic peptide, CAVIPDSTEQSDVRFSSAV (Multiple Peptide Systems, San Diego, CA) derived from the C-terminus of the human GPER1 receptor, which was supplied by E Filardo (Revankar et al., 2005). The specificity of this antibody has been shown on Western blots and in preadsorption controls (Filardo et al., 2000; Hammond & Gibbs, 2011).

Tissue preparation. Rats were perfused, and brains were prepared for immunolabelling of mPFC tissue (Fig 2F) as described previously (Almey et al., 2012; T. A. Milner, Waters, B., Robinson, D., & Pierce, J.P, 2011). Additionally, in all experiments that involved immunolabelling for ERα or ERβ, a tissue section containing the ventromedial and arcuate nuclei of the hypothalamus was included in the immunohistochemical procedure as a positive control. Abundant ERα and ERβ labeling are present in these regions (Kritzer, 2002), so the success of immunolabelling could be confirmed prior to processing the mPFC for EM.

Immunohistochemical labeling and tissue fixation and embedding. Free floating tissue sections containing the mPFC from three of the six rats were each processed for immunohistochemical localization of ERα, ERβ, or GPER1. Briefly, sections were incubated in anti-rabbit ERα (1:10,000 dilution), ERβ (1:2000) or GPER1 (1:1000) for 24 hours at room temperature, and 4 days at 4°C in 0.1% BSA in TBS. Sections were then incubated in 1) biotinylated donkey anti-rabbit immunoglobulin (IgG; diluted 1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.5% BSA in TBS, 30 minutes; 2) avidin-biotin complex (Vector, Burlingame, CA), 30 minutes; and 3) 3,3-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and H_2O_2 in TBS, 6-7 minutes. Following immunolabelling, tissue sections were fixed in osmium, embedded in plastic and sectioned and collected on grids as described previously (Almey et al., 2012).

Sections through the mPFC were examined under a Philips CM10 electron microscope with an AMT digital camera. The subcellular distribution of each ER was examined in two sections per rat; a 5,832μm^2 area of each section were counted in each section and categorized

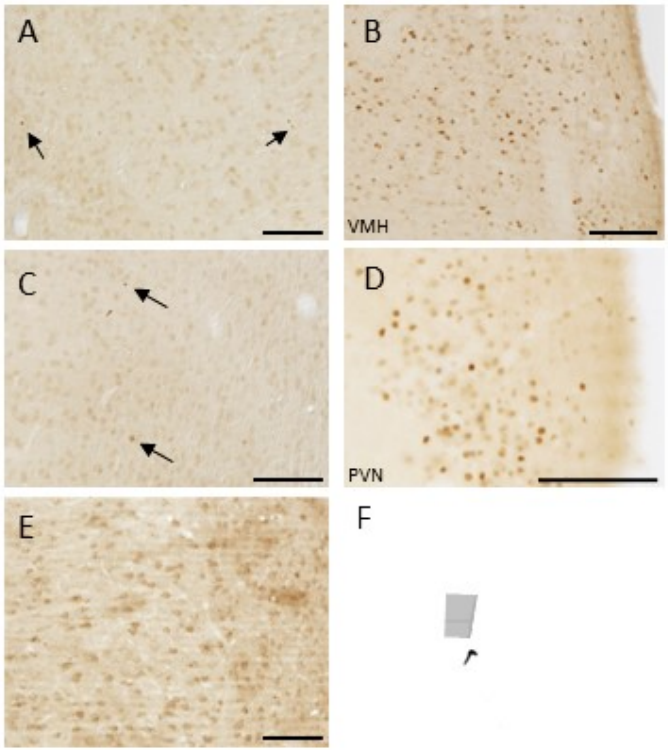

Figure 2. Light microscopic examination of ER localization in the mPFC. **A)** No nuclear or extranuclear immunoreactivity (IR) for ERα was observed. **B)** Dense ERα-IR in the ventromedial hypothalamus. **C)** No ERβ-IR was observed in the mPFC. **D)** Dense nuclear ERβ-IR in the hypothalamus. **E)** Dense extranuclear GPER1-IR is detected in the neuropil. **F)** A coronal schematic depicting the area of the mPFC (grey trapezoid) analyzed by electron microscopy. Black arrows depict nuclear labelling.

as: dendrites, dendritic spines, axons, axon terminals, or glia, using established criteria(Peters, 1991), see Almey et al. (Almey et al., 2012) for a specific description of profiles identification. The total number of labelled profiles was averaged across the three rats. Tissue selected for analysis was taken from a depth of 0.2-1.5µm from the plastic–tissue interface, and only samples thin sectioned evenly across the plastic tissue interface were included in analyses. Soma were not included in the quantification analyses, as they frequently occupy more than half of the area analyzed, reducing the overall number of ER-IR profiles observed. Final photomicrographs were generated from digital images, where brightness and contrast were adjusted using GIMP 2.8. Figures were assembled in Microsoft PowerPoint 2013.

3. Results

3.1 Experiment 1 – mPFC and memory systems bias

Histology. The study began with 32 rats, but 3 rats were eliminated because they never reached criterion, so 29 rats were included in the final analysis (n =14 for mPFC, n =15 for AC). Cannula placements in the mPFC and the AC of these remaining rats were within the target brain regions (Fig 3A, B).

Behaviour. After a microinfusion of E2 into the mPFC, 86% of rats used place memory and 14% used response memory; following microinfusions of vehicle to the mPFC 29% of rats used place memory while 71% used response memory (Fig 3A). This difference in memory use following E2 and cyclodextrin infusions was statistically significant (McNemar test, p = 0.008), demonstrating that E2 administered directly to the mPFC of female rats induced a bias towards use of a place memory. The odds ratio indicates that use of place memory was 15 times higher following an infusion of E2 than it was following an infusion of vehicle. The logit d effect size was 1.56, which demonstrates that this is a large effect (Klein, 2004).

In contrast to the findings following microinfusions into the mPFC, E2 or vehicle infusions into the AC elicited comparable behavioural effects. When E2 was infused to the AC 13% of the rats used a place memory and 87% of the rats used response memory; similarly, when vehicle was infused into the AC 20% of rats used place memory while 80% of rats used response memory (Fig 3B). Analysis with a McNemar test revealed no significant difference in memory use following infusions of E2 or cyclodextrin to the AC. The odds ratio was 0.62 indicating that

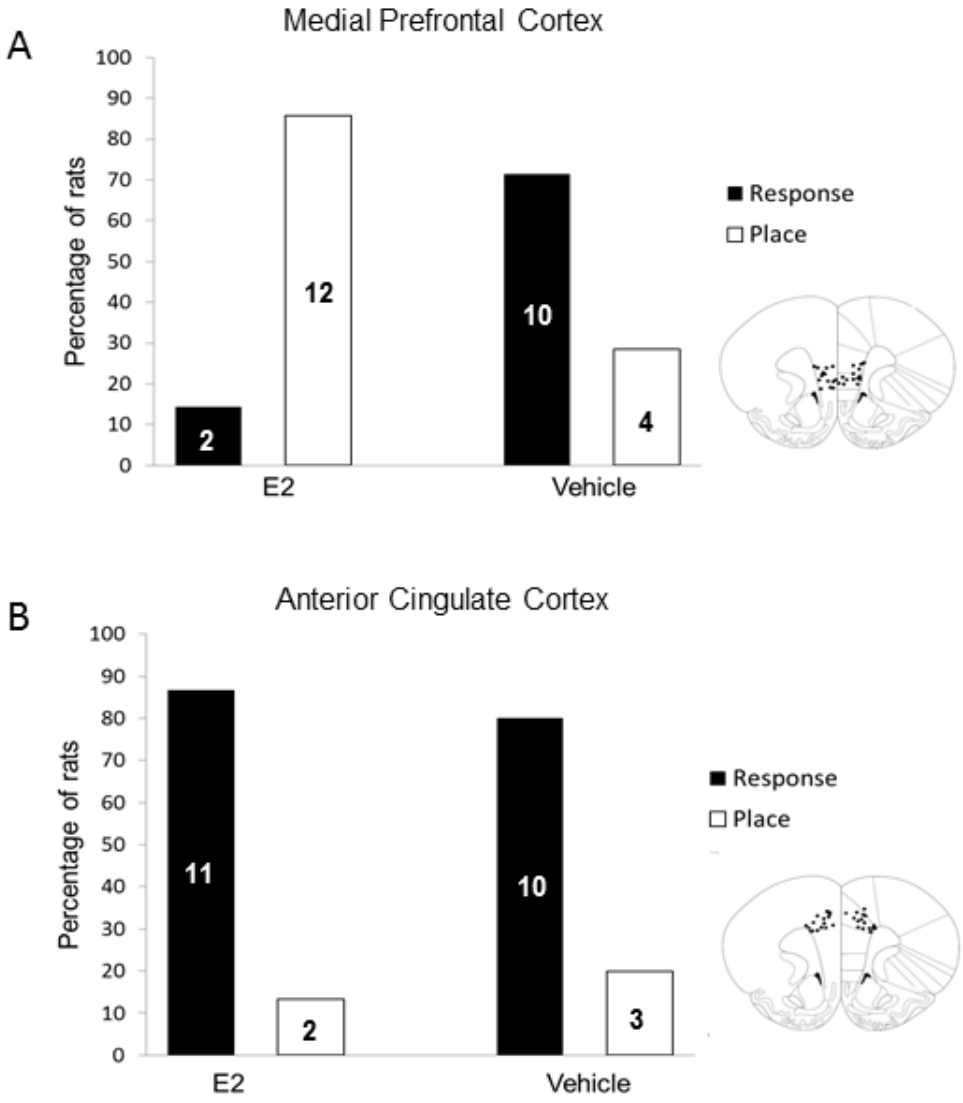

***Figure 3*.** Percentage of rats that used a place or response strategy following microinfusions of E2 or vehicle in: **A)** the medial prefrontal cortex and **B)** the anterior cingulate cortex. The number of rats per group is shown on the bar, and cannula placements are shown in the image beside the graph.

there was close to an equal chance of rats using a place and response strategy in the E2 and vehicle groups. The logit *d* effect size, was 0.28, which is a small effect (Klein, 2004).

3.2 Experiment 2 – Ultrastructural analysis of ERs in the mPFC

Light microscopy. By light microscopy, no nuclear or extranuclear ERα- or ERβ-labeling was observed in the mPFC (Fig. 2A and C). However, abundant nuclei containing ERα-IR and ERβ-IR were seen in the ventromedial and arcuate regions of the hypothalamus, indicating that immunohistochemistry was successful (Fig 2B and D). In contrast, GPER1-IR was observed in the cytoplasm, but not nuclei, of perikarya throughout the mPFC (Fig 2E).

ERα, ERβ, and GPER1 are observed primarily at presynaptic sites in the mPFC.

ERα. At the ultrastructural level, ERα-IR was present in all types of neuronal processes and glia in the mPFC (Fig. 4). Semi-quantitative analysis (Table 1) demonstrated that most ERα-IR was in axons (41.7%) and axon terminals (28.8%). In axons (<0.15 μm in diameter), ERα-IR was typically discrete and was affiliated with the plasma membrane or clusters of small vesicles (Fig 4A and C). Axon terminals had cross sectional diameters that ranged from ~0.3-0.8μm, and contained numerous small synaptic vesicles (SSVs) and occasionally mitochondria, but no dense-core vesicles. In terminals, ERα-IR was found in clusters around SSVs (Fig 4A), at the plasma membrane, and occasionally associated with mitochondrial membranes. In addition to presynaptic sites, some ERα-IR labeling was observed in dendritic shafts (~8.3%) and dendritic spines (6.7%). In the dendritic shafts, peroxidase reaction product was often affiliated with the plasma membranes and microtubules (Fig. 4D), and was occasionally observed at mitochondrial membranes. In dendritic spines, ERα-IR accumulated in the spine head, and was observed on the plasma membrane particularly near the post-synaptic density. ERα-IR was found at both pre- and post-synaptic profiles forming asymmetric synapses. Occasionally, ERα-IR axon terminals synapsed onto ERα-IR spines. Finally, ERα-IR was observed in glial profiles (14.4%; Fig. 4B).

ERβ. At the ultrastructural level ERß-IR was observed almost exclusively at extranuclear sites in neuronal and glia profiles (Fig. 5; Table 1). ERß-IR was most commonly localized in axons (28.8%) and axon terminals (29.9%). In axons, ERß-IR was typically found throughout the profiles (Fig 5B). In axon terminals, ERß-IR was observed in clusters of reaction product associated with SSV and was sometimes affiliated with the plasma membrane (Fig. 5C). ERß-IR was also in dendrites (9.6%) and dendritic spines (10.7%). ERß reaction product filled dendritic profiles but often was densest near the plasma membrane. In dendritic spines ERß-IR

Table 1

Distribution of mERs in the mPFC

Receptor		ERα	ERß	GPER1
Dendrites	**%**	8.3	8.4	12.9
	# SEM	10.7 ±0.3	5.7 ±1.2	36.3 ±2.3
Spines	**%**	6.7	9.4	6.0
	# SEM	8.7 ±0.9	6.3 ±0.9	17.0 ±2.3
Axons	**%**	41.7	25.3	36.7
	# SEM	53.7 ±4.1	17.0 ±1.5	103.7 ±6.2
Terminals	**%**	28.8	33.2	29.3
	# SEM	37.0 ±2.5	22.3 ±2.3	82.7 ±4.8
Glia	**%**	14.5	18.3	14.4
	# SEM	18.7 ±3.2	12.3 ±2.0	40.7 ±2.0
Total	**%**	100	100	100
	# SEM	128.7 ±4.3	67.3 ±2.3	282.3 ±9.2

Percentage of total immunoreactive profiles, and number of IR profiles and the corresponding standard error, observed in a ~6000 μm area of the medial prefrontal cortex, averaged across rats.

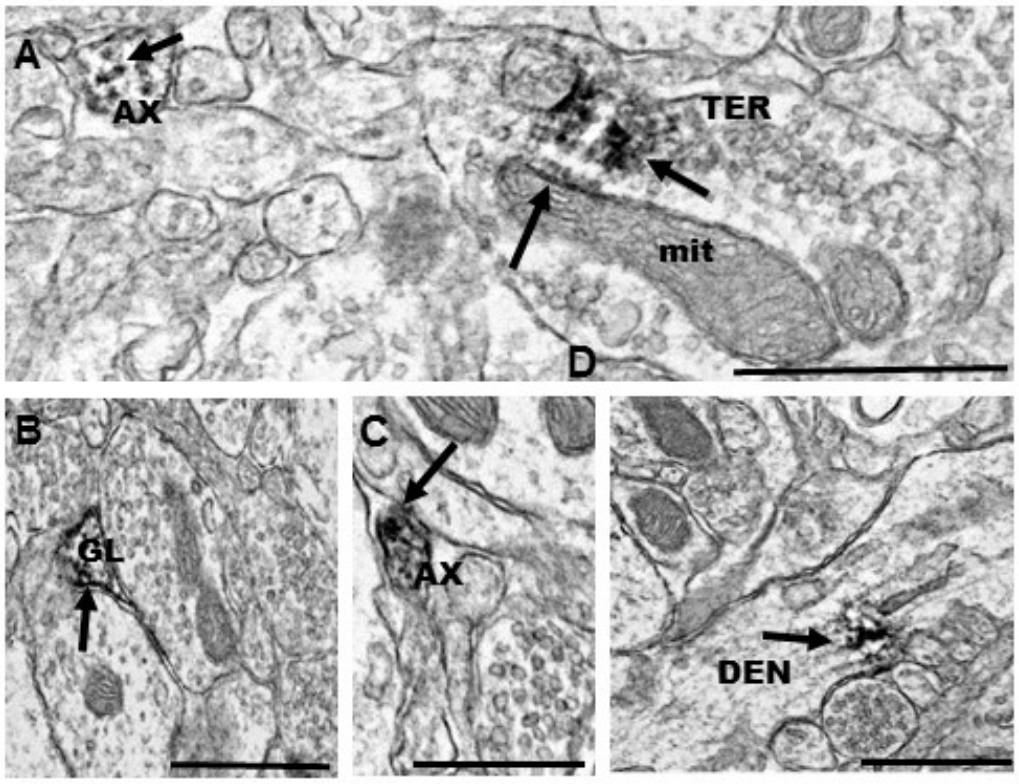

***Figure 4*. Electron micrographs showing examples of profiles containing ERα immunoreactivity (IR) in the mPFC**. These photomicrographs show IR for: **A)** ERα in an axon (AX) and in a terminal (TER), where IR is observed at small synaptic vesicles and on the membrane of a mitochondrion (mit) **B)** ERα-IR associated with the membrane of a glial cell (GL) **C)** ERα-IR filling an axon (AX) **D)** IR for ERα in a dendrite, observed at the plasma membrane and associated with microtubules. *Bar*, 500nm.

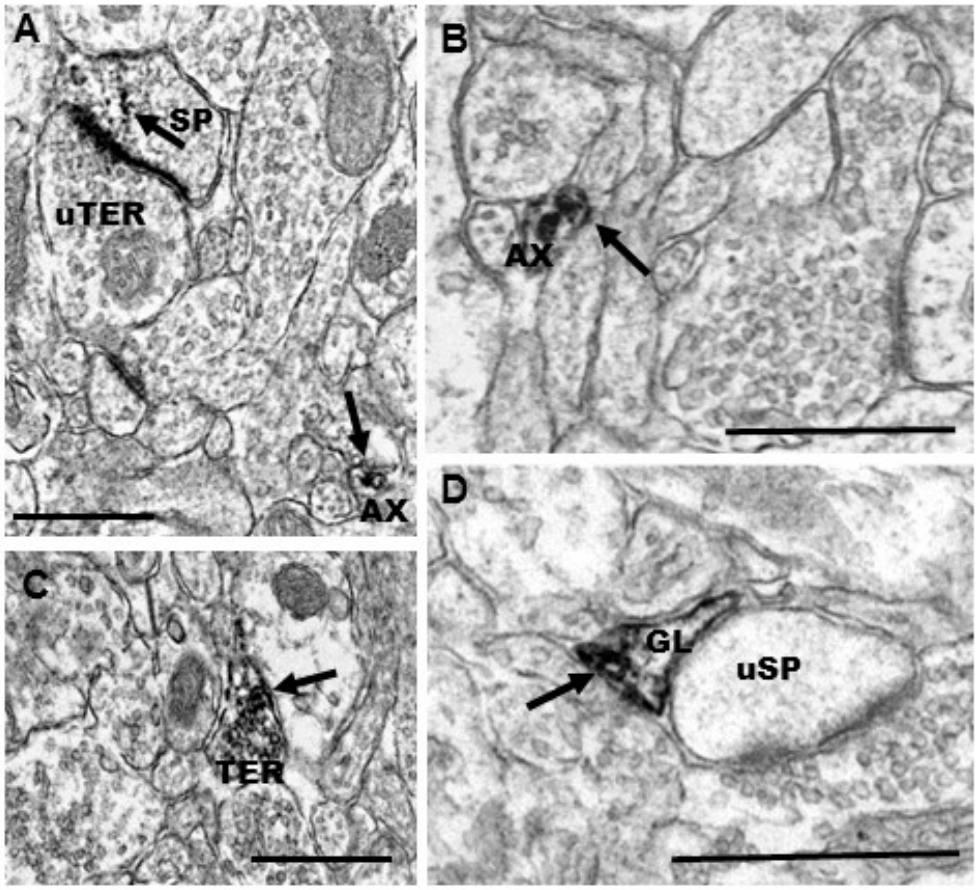

***Figure 5*. Electron micrographs showing examples of profiles containing immunoreactivity (IR) for ERβ in the mPFC.** These photomicrographs show: **A)** ERβ in an axon (AX) and in a dendritic spine (SP) that forms an asymmetrical synapse with an unlabeled terminal (uTER) **B)** ERβ immunoreactivity filling an axon profile (AX) **C)** ERβ immunoreactivity associated with vesicles and the plasma membrane of an axon terminal (TER) **D)** GPER1 in a glial cell that is in apposition to an unlabeled dendritic spine (uSP). *Bar,* 500nm.

typically accumulated in the spine head, and was frequently observed at the cell membrane near the synapse (Fig. 5A). ERß-IR was occasionally observed in the perikarya where, it was observed at the plasma membrane and associated with organelles (not shown). ERβ-IR was observed in terminals and dendritic spines that formed asymmetric synapses, but ERβ-IR terminals were not observed forming synapses with ERβ-IR spines. ERß-IR also was frequently observed in glia profile (20.9%; Fig. 5D).

GPER1. Immunoperoxidase labeling for GPER1 was observed throughout the mPFC (Fig. 6; Table 1). Like ERα and ERβ, the majority of GPER1-IR was presynaptic: axons and axon terminals accounted for 36.7% and 29.3% of the GPER1 labelled profiles, respectively. In axons, GPER1-IR was usually discrete, and often associated with the plasma membrane or small clusters of vesicles (Fig. 6C and D). In axon terminals GPER1-IR was most commonly clustered on groups of SSVs or at the plasma membrane (Fig 6A). GPER1-IR also was observed at post-synaptic sites: dendritic shafts constituted 12.8% of total GPER1 labelled profiles, and dendritic spines constituted 6.2% of the total IR profiles. In dendritic shafts, GPER1-IR was typically associated with the plasma membrane, but also was affiliated with microtubules (Fig. 6D), and mitochondrial membranes. In dendritic spines, GPER1-IR peroxidase reaction product accumulated in the spine head, and was associated with the plasma membrane, sometimes in the perisynaptic zones (Fig 6B). Neuronal perikarya with GPER1-IR were also observed. Labeling was exclusively in the cytoplasm where it was discretely affiliated with endoplasmic reticulum, mitochondria, and the plasma membrane (Fig 6C). Although GPER1-IR was observed both pre and post-synaptically, and was often observed close to the synapse in terminals and spines, it was rare for GPER1-IR terminals to synapse onto GPER1-IR spines. Finally, GPER1-IR was observed in glia profiles (14.4%); the labeling in glial cells was discrete, and was observed at the plasma membrane.

4. Discussion

An infusion of E2 to the mPFC, but not the AC, of female rats rapidly induces a bias towards the use of place memory to solve an appetitive task. Ultrastructural analyses demonstrate that ERα, ERβ, and GPER1 are all present, almost exclusively at extranuclear sites in the mPFC, providing a mechanism via which E2 in the mPFC could rapidly alter memory system use.

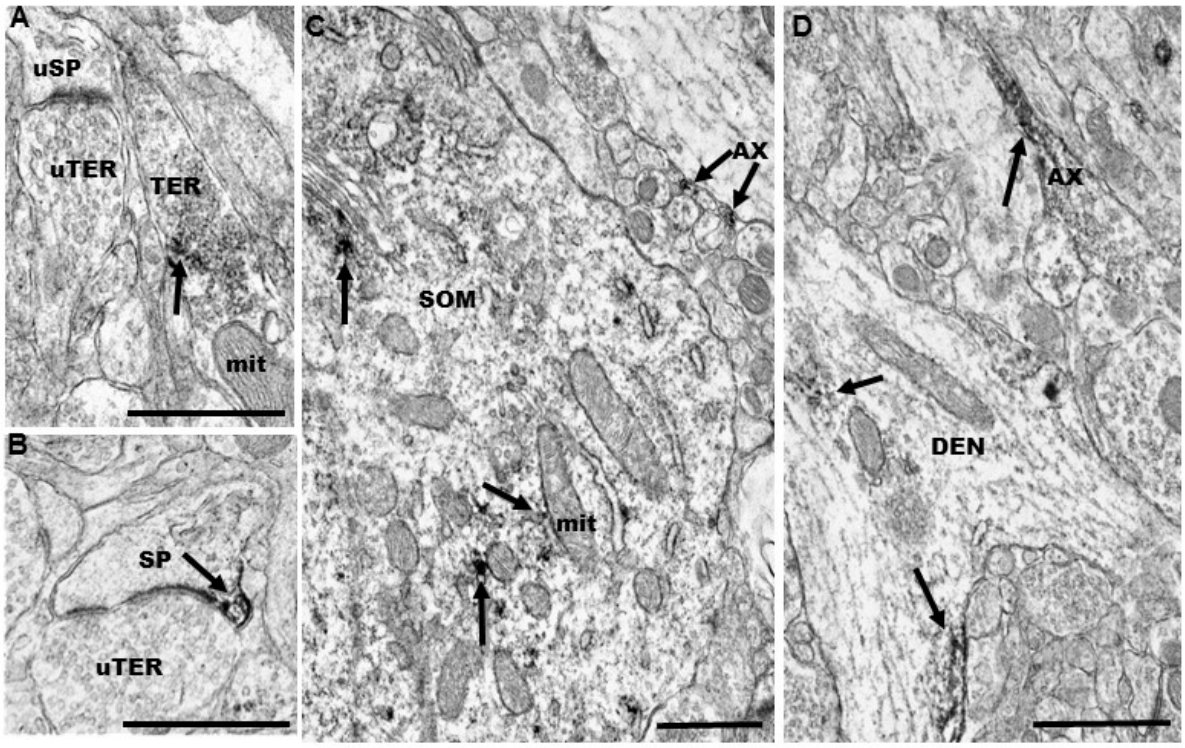

***Figure 6*. Electron micrographs showing examples of profiles containing immunoreactivity (IR) for GPER1 in the mPFC.** These photomicrographs show IR for: **A)** GPER1-IR associated with small synaptic vesicles in a terminal (TER) that is adjacent to an unlabeled terminal(uTER) and spine (uSP) that form an asymmetric synapse **B)** GPER1-IR associated with the plasma membrane of a dendritic spine that is forming a synapse with an unlabeled terminal (uTER) **C) F)** GPER1 in a soma (SOM) where it is localized to the endoplasmic reticulum and the membrane of mitochondria (mit), and IR for GPER1 in two axons (AX) **G)** GPER1 in an axon (AX) and in a dendrite (DEN),where it is associated with microtubules and the cell membrane. *Bar*, 500nm.

4.1 Experiment 1 – mPFC and memory systems bias

Without additional intracortical E2 administration, rats in both the mPFC and AC groups had chronic low E2 serum levels, via subcutaneous implants, that are typically associated with a bias towards use of response memory (Korol & Kolo, 2002; Korol et al., 2004; M. G. Quinlan et al., 2008). Following injection of the vehicle, cyclodextrin, into either the mPFC or the AC, rats still predominantly used a response strategy (71% and 80%, respectively). This finding agrees with previous studies, which show that response memory was used by 73% of OVX female rats with silastic capsules maintaining a low level of E2 (M. G. Quinlan et al., 2008) and 71% of female rats in the estrus phase of the cycle, when E2 is low (Korol et al., 2004).

Estrogens in the mPFC bias female rats towards use of place memory. Interestingly, E2 administered directly to the mPFC biases female rats towards use of place memory, which offers new insight into how systemic E2 influences multiple memory systems. In this experiment 86% of rats that received an infusion of E2 to the mPFC used a place strategy, which provides strong evidence that systemic E2 is acting, at least in part, in the mPFC to elicit this bias towards place strategy use. This finding parallels previous research demonstrating that high serum levels of E2 are associated with a bias towards use of place memory (Korol et al., 2004; M. G. Quinlan et al., 2008). The infusion of E2 to the AC did not alter memory system use, with 87% rats using response memory following an infusion of E2. Since E2 administration to the AC has no effect on multiple memory system use, this suggests that E2-induced changes in place memory use are specific to the mPFC, and do not generalize to other regions of the frontal cortex. However, it is recognized here that estrogens also act in the hippocampus and STR to influence memory system bias (Zurkovsky et al., 2007; Zurkovsky et al., 2011). Since there are reciprocal connections between the regions there may be interactions between the E2 effects in the mPFC, STR, and hippocampus, but this remains unclear. Additionally, the time between the microinfusion of E2 and testing was ~10 minutes, so the effects of E2 in the mPFC on memory system bias are likely be rapid, resulting from binding at membrane-associated E2 receptors.

When the results of the present study are considered in the context of previous research, it seems possible that E2 in the mPFC influences strategy use by altering dopamine transmission in this region. Dopamine projections to the mPFC originate from the ventral tegmental area and to a lesser extent, from the substantia nigra (Heidbreder & Groenewegen, 2003). Dopamine transmission in the mPFC is influenced by E2, such that higher serum levels of E2 are associated

with lower basal levels of dopamine in the mPFC (Dazzi et al., 2007) and lower levels of dopamine in mPFC homogenate (Dupont et al., 1981; Luine, Richards, Wu, & Beck, 1998). Additionally, findings from this lab indicate that female rats with low levels of E2 switch from a response memory to a place memory when administered either a D1 or D2 receptor antagonist, either systemically (Quinlan et al., 2008) or directly into the mPFC (Quinlan, Radiotis, Lachapelle, Caisse & Brake, 2014). This experiment parallels such findings, since infusions of E2 to the mPFC also induce a switch from response to place strategy in female rats with low systemic levels of E2. Together, these findings are consistent with a model of strategy use in which changes of dopamine transmission in the mPFC, either via presynaptic E2-induced inhibition of dopamine availability or via dopamine receptor antagonist, bias rats toward the use of place strategy.

4.2 Experiment 2 – Ultrastructural analysis of ERs in the mPFC

These experiments demonstrated that, at the ultrastructural level, ERα, ERβ, and GPER1 are localized to extranuclear sites in neuronal and glial profiles in the mPFC of female rats. Although ERs are observed at all neuronal profiles, the majority of ERs are observed on axons and terminals, suggesting that estrogens alter transmission in the mPFC via presynaptic mechanisms. Additionally, when results for the three ERs are considered together it is clear that GPER1 is the most common ER in the mPFC of female rats, since GPER1 is twice as abundant as ERα, and 4 times more abundant than ERβ (see Table 1). This implies that the effects of E2 in the mPFC occur predominantly through binding at GPER1, although binding at ERα and ERβ would also affect transmission in the mPFC. These microscopy results contribute to an explanation of how E2 rapidly alters transmission in the mPFC to affect memory system bias.

Methodological Considerations. To determine whether ERα, ERβ, and GPER1 are found in the mPFC, the present study used an immunoperoxidase label and preembedding methods, which result in excellent cellular morphology that allows for discrete subcellular localization of antigens (Leranth C., 1989). To ensure that any differences in number of labelled profiles were not due to differences in antibody penetration or sample size, all tissue samples analysed for quantification were identical in size and taken from near to the plastic/tissue interface. This methodology tends to underestimate the absolute number of peroxidase labelled profiles (Leranth C., 1989). IR for ERα, ERβ and GPER1 are discrete, so the absence of ER labeling within cellular profiles does not demonstrate that these profiles lack ERs. Thus, the

quantification analyses presented here are conservative, and likely underestimate the actual numbers of profiles containing these ERs.

ERα, ERβ and GPER1 are observed exclusively at extranuclear sites in the mPFC. ERα, ERβ, and GPER1 in the mPFC are exclusively localized to extranuclear sites, and are observed at the plasma membrane in all types of neuronal profiles and glial cells. This finding contrasts some previous light microscopy and in situ hybridization studies that observe little ERα and ERβ, or observe ERα and ERβ exclusively at nuclear sites (Cardona-Gomez et al., 2000; Kritzer, 2002; Shughrue & Merchenthaler, 2001). However, this study is in agreement with other research that demonstrates immunolabelling for ERα in the mPFC of monkeys and rats (Montague et al., 2008; Wang et al., 2010), and moderate levels of ERβ mRNA in the mPFC of mice (Mitra et al., 2003). The difference between the present study and previous studies is likely because of the greater sensitivity and resolution of EM; in this experiment, light microscopy was not sufficient to observe any extranuclear ERα or ERβ, but EM allowed for the detection of discrete membrane-associated ERα and ERβ-IR in the mPFC. To our knowledge this is the first time GPER1 has been localized to the mPFC of the rat. At the ultrastructural level, GPER1 is observed at the plasma membrane and in the cytoplasm of various neuronal profiles, corresponding to previous research examining the distribution of GPER1 (Filardo et al., 2006; Filardo & Thomas, 2012; Matsuda et al., 2008).

All ERs are predominantly localized to presynaptic sites in the mPFC. At the ultrastructural level, the highest proportions of ERα, ERβ, and GPER1-IR profiles are in axons and terminals, which parallels previous findings in primates (Wang et al., 2010). This indicates that estrogens in the mPFC likely alter neurotransmission via pre-synaptic mechanisms, such as vesicle formation, immobilization, and/or release of neurotransmitter from the terminal. ERs observed in axons might reflect transportation of these receptors from the soma to the terminal, but these receptors may also have effects on protein transport or the transduction of electrochemical signals (Cheung, 1990; Verdier et al., 2003). Additionally, the presynaptic receptors observed in terminals may be important in the local control of transmitter release, as estrogens have been shown to decrease dopamine availability in the mPFC (Dazzi et al., 2007). ER-IR is observed exclusively at extranuclear sites in the mPFC, which is in congruence with previous findings that have localized this receptor to extranuclear sites in other brain regions, such as the hippocampus and STR of rodents (Almey et al., 2012; T. A. Milner et al., 2001) and

the PFC of primates (Wang et al., 2010). Binding at these receptors on the plasma membrane could rapidly alter transmission in the mPFC, which provides a possible mechanism for estrogens' rapid effects on multiple memory system bias.

In addition to ERs being localized to presynaptic sites, they were also observed at post-synaptic sites, on dendrites and dendritic spines, indicating that estrogens in the mPFC also have some post synaptic effects on transmission in the mPFC. Although ERβ was most prevalent in presynaptic profiles, it is interesting that 11% of ERβ was localized to dendritic spines, and this labeling in spines was often dense (Fig 3D), while ERα and GPER1 labeling in spines was only half of that observed with ERβ (~6%). This suggests that binding at ERβ may also have post-synaptic effects in the mPFC, such as altering the cells permeability to ions or affecting the activity of second messenger cascades.

ERs are localized to glia and to mitochondrial membranes. GPER1, ERα, and ERβ are all localized to the plasma membrane of glial cells and mitochondrial membranes in the mPFC. This parallels observations from our previous study examining ER distribution in the STR (Almey et al., 2012). Estrogens are known to mediate glial-induced neuroprotection (Arevalo et al., 2010; Liu et al., 2011), in part through binding at GPER1 (Liu et al., 2011). Thus, the localization of all three ERs to glia contributes to an explanation of how estrogens affect glial-mediated neuroprotection. Estrogens have also been implicated in mitochondrial functioning and cellular metabolism (Araujo et al., 2008; Razmara et al., 2008). The observation of ERs on mitochondrial membranes provides a mechanism for estrogen-induced alterations in cellular metabolism. Additionally, GPER1 is observed at the endoplasmic reticulum in the mPFC, paralleling findings in COS7, HEC50, and CHO cell cultures (Otto et al., 2008), and the hippocampal formation (Funakoshi et al., 2006; Matsuda et al., 2008). GPER1 is likely localized to this organelle because regulatory steps in the biosynthesis of GPER1 occur at the endoplasmic reticulum (Filardo & Thomas, 2012).

These ultrastructural findings contribute to an explanation of the mechanisms via which E2 in the mPFC biases female rats towards use of place memory. ERs in the mPFC were observed almost exclusively at extranuclear sites, indicating that estrogens would have rapid effects on neurotransmission in the mPFC. This corresponds to the behavioural findings presented here, that E2 acts rapidly in the mPFC to induce a bias towards use of place memory to navigate an environment. The majority of ERα, ERβ, and GPER1 are observed on axons and

terminals in the mPFC, and it is possible these axons and terminals are dopaminergic. It is hypothesized that the E2-induced shift towards used of place memory is caused by E2 altering dopaminergic transmission in the mPFC. This would provide a mechanism via which systemic estrogens could alter dopamine availability to affect many behaviours, including multiple memory system bias. Dual labeling studies should be conducted to determine whether these ERs are in fact localized to dopaminergic neurons in the mPFC.

CHAPTER 6:
GENERAL DISCUSSION

The behavioural experiments in this thesis demonstrate that estrogens affect some dopamine-dependent cognitive processes and facilitate the behavioural effects of the antipsychotic drug HAL. The experiments in Chapter 2 of the thesis examined the effects of no, low and high E2 replacement, alone and in combination with HAL, on LI. Results confirmed the hypotheses that E2 replacement abolishes LI in female rats, and that male rats perform similarly to females receiving no E2 replacement. Additionally these experiments also confirmed the hypothesis that estrogens facilitate the effects of HAL to restore LI. In Chapter 3 these findings were extended to two other cognitive processes, perseveration and reversal learning. The results of Chapter 3 partially supported the hypotheses; unexpectedly E2 replacement alone has no effect on perseveration or reversal learning in AMPH sensitized female rats, but E2 replacement facilitates HAL to reduce perseveration and the latency to achieve the reversal learning criterion. Finally, the behavioural experiment in Chapter 6 confirmed the hypothesis that an infusion of E2 into the PFC biases female rats towards use of place memory to navigate in their environment. This finding is one of the first to demonstrate that binding at mERs rapidly alters behaviour. The behavioural experiments in this thesis provide evidence for both long-term and rapid effects of estrogens on some dopamine-dependent cognitive processes. Additionally, these experiments demonstrate that estrogen facilitate the effects of HAL, which has implications for the treatment of schizophrenia in females.

The electron microscopy experiments in this thesis provide visual confirmation that ERs, ERα, ERβ, and GPER1, are observed at extranuclear sites and on glia in the terminal regions of the mesocorticolimbic and nigrostriatal pathways. In Chapter 4, it was hypothesized that ultrastructural analysis would observe mERs in the STR; the results of these experiments validated this hypothesis, demonstrating that all three ERs are observed exclusively at extranuclear sites in the STR. The results of the dual labelling experiments in Chapter 4A did not fully support the hypotheses, as mERs are not localized to dopaminergic neurons in the STR. However, these dual labelling studies support the hypothesis that a proportion of ERs are localized to cholinergic neurons in the STR, and the experiment in Chapter 4B confirmed the hypothesis that mERs are localized to GABAergic neurons in the STR. Chapter 5 examined the distribution of ERs in the NAc Core and Shell, validating the hypothesis that there are mERs in the NAc. These experiments provide limited support for the hypothesis that mERs in the NAc are localized to dopaminergic neurons, since a low proportion of mERs are observed in

catecholaminergic neurons, and confirmed the hypothesis that mERs are localized to GABAergic neurons in the NAc Core and Shell. Finally, the electron microscopy experiment in Chapter 6 used the same immunolabelling techniques at Chapters 4 and 5 to examine the ultrastructural distribution of mERs in the PFC; this experiment validated the hypothesis that there are mERs in the PFC, providing a mechanism for the rapid effect of on multiple memory system use in this region. The electron microscopy experiments in this thesis provide the first evidence for mERs in the terminal regions of the mesocorticolimbic and nigrostriatal pathways, providing a potential mechanism for some of the effects of estrogens on dopamine transmission and dopamine-dependent behaviours. These experiments correspond to previous light microscopy findings that observed moderate levels of nuclear ERs in the substantia nigra and ventral tegmental area, and low levels of nuclear labeling in the STR, NAc, and PFC (Brailoiu et al., 2007; Hazell et al., 2009; Mitra et al., 2003; Shughrue & Merchenthaler, 2001). The cognitive effects of estrogens likely result from a combination of the long-term effects resulting in changes in protein expression, via nuclear ERs and mERs, and the rapid effects resulting in changes in membrane permeability and vesicular trafficking, via mERs.

1. The effects of estradiol on cognition

The experiments in this thesis examined the effects of E2 on cognition in OVX female rats. More specifically, four cognitive processes were examined in this thesis: selective attention, multiple memory system bias, reversal learning, and perseveration. The effects of E2 on these four measures of cognition varied, as higher levels of estrogens reduced selective attention and biased female rats towards use of place memory to navigate their environment, but had no effect on perseveration or reversal learning. There are two possible reasons for these varied effects of E2 on cognition.

The most likely explanation for the differing effects of E2 on different cognitive processes observed in this thesis is AMPH sensitization; all rats in the reversal learning and perseveration studies were repeatedly administered amphetamine prior to testing, while rats in the LI and memory system bias experiments were not. This change in experimental protocol was made following the LI study to increase the ecological validity of the reversal learning and perseveration experiments, since AMPH sensitization of locomotor activity induces some of the cognitive symptoms associated with schizophrenia (Featherstone et al., 2007). AMPH

sensitization of locomotor activity results in lasting changes in dopamine transmission, which are hypothesized to be responsible for the behavioural changes in rodents that parallel the cognitive symptoms of schizophrenia. Briefly, sensitization to psychostimulant drugs increases stimulated, but not basal, extracellular dopamine levels (Becker, 1990a; Becker & Rudick, 1999; Thompson & Moss, 1994). Similarly, repeated administrations of AMPH result in increased stimulated glutamate in the NAc and STR (McFarland, Lapish, & Kalivas, 2003). There is some evidence suggesting that repeated administrations of AMPH are associated with stimulated extracellular dopamine in the PFC, which could contribute to the increase in glutamate in the NAc (for review see Pierce & Kalivas, 1997). Since AMPH has robust and long-lasting effects on dopamine transmission in the NA, STR, and PFC, this may mask more subtle changes in dopamine caused by E2 replacement. In sum, in the experiments presented here, E2 affected selective attention and multiple memory systems, but not perseveration or reversal learning, potentially because all rats in the perseveration and reversal learning experiments were amphetamine sensitized.

A second reason why high E2 replacement only affected some of the cognitive measures examined in this thesis is that estrogens' neurobiological effects differ between brain regions. Previous research, including the results of this thesis, suggests that estrogens may have greater effects in the PFC than they do in the NAc or STR. Findings from multiple studies indicate that there are higher levels of both nuclear and membrane associated receptors in the PFC than in the NAc or STR (Almey et al., 2014; Almey et al., 2012; Hazell et al., 2009; Mitra et al., 2003; Montague et al., 2008; Shughrue et al., 1998), suggesting that estrogens would elicit greater effects on transmission in the PFC. This is of interest because evidence suggests that the PFC plays a critical role in multiple memory system bias, switching between the different memory systems (Almey et al., 2014; Dahmani & Bohbot, 2015; Rich & Shapiro, 2009). Additionally, the PFC is also important for LI, as lesions of the orbitofrontal cortex (Schiller & Weiner, 2004), depletion of catecholamines in the PFC (Nelson, Thur, Marsden, & Cassaday, 2010), and reduced transmission at the $GABA_A$ receptor in the PFC (Piantadosi & Floresco, 2014) all disrupt this behaviour. In contrast, deficits in reversal learning caused by excessive perseveration are mediated more by the STR and NAc (Ersche et al., 2011), and the PFC to a lesser extent (Mala et al., 2015). Thus, the E2 replacement used in this experiment could have affected LI and multiple memory system bias because these behaviours are more susceptible to disruptions in

prefrontal cortical transmission, and E2 theoretically has greater effects on transmission in the PFC.

Despite differing effects of E2 on the cognitive processes assessed in this thesis, E2 consistently facilitates the effects of HAL, increasing HAL's efficacy to reduce deficits in LI, reversal learning, and perseveration. Currently there is no known mechanism for the interaction between E2 and HAL, but the effects of E2 on the distribution and affinity state of D2 receptors likely contribute. HAL is an antagonist for the D2 receptor, meaning it binds to this receptor, preventing dopamine from binding to activate the receptor. Ovariectomy results in significant decreases in D2 receptor binding in the NAc and STR, which are prevented/recovered by E2 replacement (Landry, Levesque and Di Paolo, 2002; Le Saux, Morissette and Di Paolo, 2006). Therefore, E2 could be facilitating the effects of HAL by maintaining D2 receptors, providing binding sites for HAL, allowing this drug to elicit its effects on dopamine transmission. A second potential mechanism for the interaction between E2 and HAL are E2-induced effects on the affinity state of the D2 receptor. D2 receptors are reported to have a high affinity and low affinity state; the low affinity state is functionally inactive, meaning that binding at the receptor has no effect (Samaha, Seeman, Stewart, Rajabi and Kapur, 2007). Previous research indicates that E2 replacement significantly increases the proportion of D2 receptors in the low affinity state (Levesque and Di Paolo, 1988); experiments are currently underway to confirm that estrogens do indeed alter the affinity state of these receptors. E2 replacement may decrease the number of D2 receptors in the high affinity state, essentially having the same effect as HAL, reducing the number of D2high receptors available to bind dopamine and alter dopamine transmission. Furthermore, it is possible that E2-HAL interactions occur via a combination of these two mechanisms, with E2 maintaining levels of D2 providing binding sites for HAL, but decreasing the affinity state of the D2 receptor to decrease the effects of dopamine binding at these receptors.

2. Clinical implications

The experiments in this thesis were run exclusively with rodents, however, they do allow for some speculation surrounding the treatment of schizophrenia in women. The results of the LI, perseveration, and reversal learning experiments suggest that estrogens increase the efficacy of HAL to improve some of the cognitive deficits associated with schizophrenia. In fact, the

optimal dose of HAL differed across estrogen replacement groups, suggesting that plasma estrogen levels should be considered when determining doses of antipsychotic drugs. Clinical studies demonstrate that HAL and other antipsychotic medications reduce positive symptoms of schizophrenia in women more when administered in conjunction with oral or transdermal E2 (Akhondzadeh et al., 2003; Kulkarni et al., 2014; Kulkarni, et al., 2012). The experiments in this thesis suggest that adjunctive estrogen treatments may also facilitate antipsychotic medication to ameliorate cognitive symptoms of schizophrenia. Antipsychotic treatments are not particularly effective at treating the cognitive symptoms of schizophrenia (Keefe, Silva, Perkins, & Lieberman, 1999), and these cognitive symptoms are the best predictor of functional outcomes (Bowie & Harvey, 2006), so any adjunct treatment that improves treatment of the cognitive symptoms of schizophrenia should be implemented in clinical treatment regimes. Adjunctive administration of E2 is effective in both female and male patients (Kulkarni et al., 2011; Kulkarni, Hayes, et al., 2012), and improves symptoms in treatment resistant individuals (Kulkarni et al., 2014), suggesting that E2 could improve the response to antipsychotic medication for the majority of individuals with this disorder. However, the results presented in this thesis demonstrate that the optimal dose of HAL differed across estrogen replacement groups, and suggest that the combination of HAL and E2 dose must be carefully titrated, since excessive doses can have detrimental cognitive effects.

Since preclinical and clinical studies suggest that estrogens facilitate the effects of antipsychotic drugs, this should be implemented in treatment practices. The addition of estrogen administration to schizophrenic women would be particularly beneficial, since antipsychotic treatments cause anovulation, infertility, and significant reductions in plasma levels of estrogens (for review, see Bargiota, Bonotis, Messinis, & Angelopoulos, 2013). Briefly, the common feature of antipsychotic drugs is antagonism of D2 receptors throughout the body, including D2 receptors on lactotroph cells in the pituitary. Dopamine binding at these pituitary D2 receptors provides tonic suppression of prolactin release; when antipsychotic drugs block these D2 receptors this leads to an increase in prolactin levels, which disrupt reproductive endocrine systems (Dickson & Glazer, 1999). Consequently, it is relatively common for females prescribed antipsychotics to have low plasma levels of estrogens, meaning that their medication is less effective than it could be if these women had normal reproductive cyclicity. There is an impetus

to translate the preclinical findings presented here into clinical practice, since adjunctive treatment with estrogens could significantly improve the efficacy of antipsychotic medications.

3. mERs in the STR, NAc, and PFC

The experiments in this thesis demonstrate that there are mERs localized to the STR, NAc, and PFC. The mERs in the hippocampus, STR, NAc, and PFC are almost never observed using light microscopy, indicating that electron microscopy is a better technique for visualizing mERs in neuronal tissue. These ultrastructural analyses complement previous light microscopy and *in situ* hybridization experiments; together they map the distribution of nuclear ERs and mERs, providing a more complete picture of how estrogens affect neurotransmission through long-term and rapid mechanisms.

The results of the electron microscopy experiments in Chapters 4-6 provide the first descriptions of the ultrastructural distribution of mERα, mERβ, and GPER1 in these dopamine-innervated regions. These results indicate that these receptors have very similar distributions in the NAc Core and Shell, suggesting a homogeneous distribution of receptors throughout the NAc. Furthermore, the distribution of mERα and GPER1 were very similar in the STR and NAc, suggesting that these receptors are distributed fairly homogeneously across these two regions. Technically the STR and NAc are both part of the striatum, which is subdivided into the dorsal striatum (STR aka. caudate putamen) and the ventral striatum (NAc; for review see Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). There are notable differences between these regions of the striatum, including the fact that dopamine innervation to the STR and NAC come from separate populations of dopamine neurons, the substantia nigra and ventral tegmental area, respectively (Voorn et al., 2004). However, the results presented in this thesis indicate that the distribution of mERs is relatively consistent throughout the striatum. The one exception to this homogeneity is the distribution of mERβ, which is higher in the NAc than in the STR. In contrast the distribution of mERs differs between the striatum and the PFC; in the PFC GPER1 is observed more frequently and mERα is observed less frequently than in the STR and NAc. Additionally there are differences in mER distribution between striatum and hippocampus, as electron microscopy research in the hippocampus indicates a greater proportion of mERs are observed at postsynaptic sites. Thus, the distribution of mERs is not consistent in all regions receiving dopamine afferents, although distribution in the STR and NAc are similar.

mERα, mERβ, and GPER1 are relatively ubiquitous in all brain regions examined in this thesis, in that they are observed at all types of neuronal profiles (dendrites, dendritic spines, axons and axon terminals) and glial cells in the STR, NAc Core and Shell, and PFC. However, in all three of these brain regions the majority of mERs are at presynaptic sites, either axons or axon terminals, indicating that estrogens primarily affect presynaptic transmission in these regions. An example of this can be seen in Figure 1, which depicts the distribution of mERα, mERβ, and GPER1 in the PFC; the majority of all three of these receptors are localized to presynaptic profiles, either axons or axon terminals. Little is known about the specific effects of binding at mERs in presynaptic neuronal profiles. However, binding at mERα associated with small synaptic vesicles in terminals of the hippocampus was shown to mobilize transportation of vesicles to the synapse (Hart et al., 2007). Binding at mERα, mERβ, and GPER1 in the STR, NAc, and PFC could have the same effect as in the hippocampus, promoting the trafficking of vesicles to the synapse in terminals. Additionally, these receptors are positioned to affect propagation of axon potential and the release of transmitter into the synaptic cleft; future studies should examine how binding at mERs alters presynaptic transmission.

Contrary to our initial hypothesis, mERs in the STR and NAc are likely not localized to dopaminergic neurons. In the STR almost no colocalization is observed between mERα or GPER1 –IR profiles and TH immunoreactivity, and in the NAc Core and Shell a small proportion of ERα and GPER1 –IR profiles also contain TH. The TH containing neurons in the NAc could be dopaminergic, so it is possible that a small proportion of mERs are localized to dopamine neurons in this region, but TH containing neurons in the NAc could also be noradrenergic. Since the distribution of mERs in the STR and NAc is relatively homogeneous, and mERs are not localized to dopaminergic neurons in the STR, it is hypothesized here that the

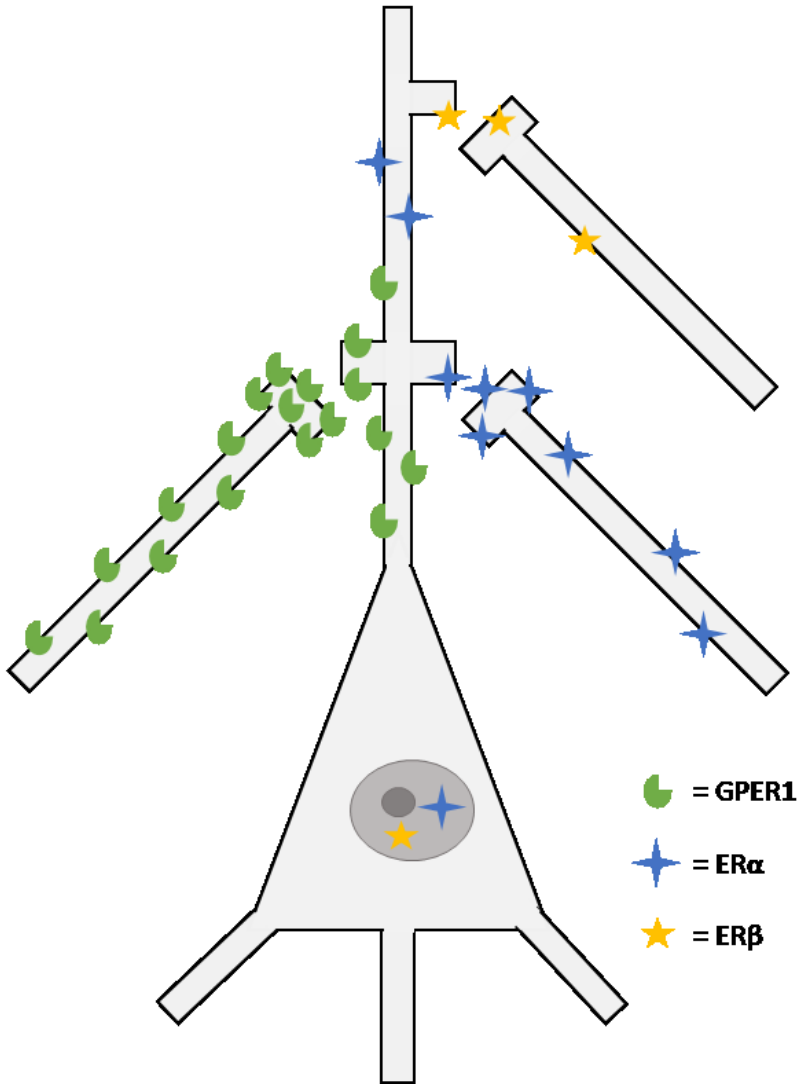

Figure 1. Representation of estrogen receptor localization to pre- and post-synaptic profiles in the prefrontal cortex. Estrogen receptors were most commonly localized to axons and terminals in the prefrontal cortex, depicted alongside the pyramidal neuron, and were also observed in dendrites and dendritic spines at lower levels, depicted on the apical dendrite of the pyramidal neuron. Low levels of nuclear labeling for ERα and ERβ were observed via light microscopy.

TH neurons in the NAc that contain mERs are noradrenergic. Further research is needed to confirm this using specific immunomarkers for dopamine and noradrenaline neurons, such as dopamine-beta-hydroxylase or phenylethanolamine-N-methyltransferase, respectively (Lorang, Amara, & Simerly, 1994). Regardless, the proportion of mERs in TH-IR neurons in the NAc is very low, suggesting that mERs in the NAc and STR have minimal effects on dopamine through binding at receptors on catecholaminergic neurons.

The results of the experiments presented in this thesis suggest that the majority of mERα and GPER1 are localized to GABAergic neurons in the STR and NAc (Fig 2 and Fig 3). This distribution of mERs suggests that estrogens in the STR and NAc indirectly affect dopamine transmission by altering GABAergic transmission in these regions. There are a select few studies that support this hypothesis. As discussed above, estrogens rapidly affect the transportation of vesicles to the synapse in the hippocampus; this is observed in GABAergic neurons, demonstrating that binding at mERs affects presynaptic GABAergic transmission in the hippocampus (Hart et al, 2007). Additionally, a systemic injection of E2 rapidly decreases GABA availability in the STR (Hu et al., 2006; Schultz et al., 2009), which indicates that binding at mERs in the STR could decrease GABA availability. Multiple studies have shown that antagonizing GABA in the STR and NAc increases tonic DA availability (Adermark et al., 2011; Smolders et al., 1995; Whitehead et al., 2001), which suggests that E2-induced decreases in GABA could cause the E2-induced increase in dopamine availability observed in these regions (Becker 1990; Becker, 1999; Becker and Rudick, 1999). It is not clear how changes in GABA availability in the STR and NAc affect dopamine availability; one theory is that GABA binds to presynaptic receptors on dopaminergic terminals in the STR/NAc, increasing dopamine release (Adermark et al., 2011). Alternatively, changes in GABAergic transmission in the STR/NAc could alter activity in glutamatergic projections from the STR/NAc to the substantia nigra/ventral tegmental area, causing increased activity in the substantia nigra leading to an increase in dopamine release. These E2-induced changes in GABA affect rotational behavior (Schultz et al., 2009), which is known to be dopamine-dependent, providing the first evidence that estrogens affect dopamine-dependent behaviours by decreasing GABA transmission.

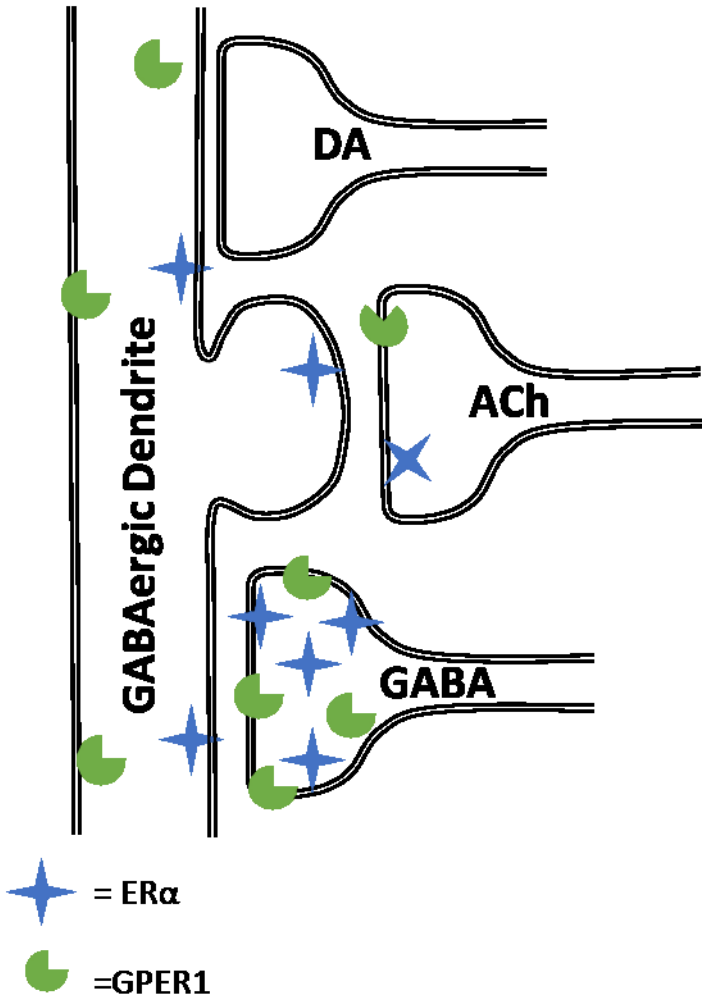

Figure 2. Representation of estrogen receptor localization within pre- and post-synaptic profiles in the dorsal striatum. Although estrogens are known to increase dopamine release in this area, almost no estrogen receptors were observed on dopamine terminals. Thus, estrogens are likely to affect dopamine release via changes in presynaptic transmission of GABA or cholinergic neurons.

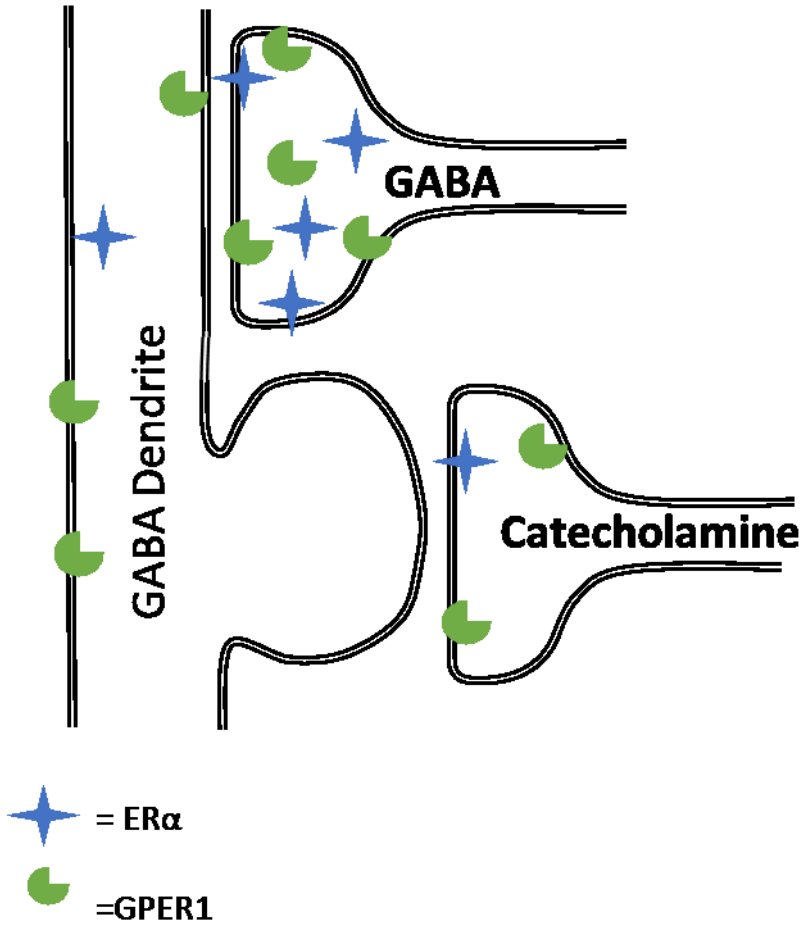

Figure 3. Representation of estrogen receptor localization in pre- and post-synaptic regions in the nucleus accumbens. There are low levels of estrogen receptors associated with dopaminergic terminals, so estrogens could affect dopamine transmission directly by binding at these receptors. Additionally, a moderate proportion of ERα and GPER1 are observed in GABAergic terminals and dendrites; estrogens could indirectly affect dopamine release through pre- or post-synaptic changes in GABAergic transmission.

4. The role of mERs in E2-induced changes in cognition

There is an abundance of research indicating that ERs are associated with the membrane, as these receptors have been localized to the membrane using fractionation (Pietras and Szego, 1980), can be activated by E2-BSA that does not cross the cell membrane (Zheng and Ramirez 1997), and occur in the presence of transcription blockers (Kelly et al 1980, Minami et al 1990, Nabekura et al 1986). Additionally, there is strong evidence that estrogens can have rapid effects on cellular transmission, initiating second messenger cascades, increasing intracellular CA^{2+} (Coleman and Smith 2001, Fu and Simoncini 2008, Yang et al 2008), and activating mGluR receptors (Meitzen et al 2013). However there is still relatively little evidence for rapid behavioural effects of estrogen.

The vast majority of studies examining the cognitive effects of estrogen administer hormone replacement 12-24 hours prior to behavioural testing, and in experiments with naturally cycling rats phase of the cycle is typically determined ~12hours prior to testing. This has been the common practice, since estrogens were thought to have long-term effects, requiring long periods of time to occur (i.e. hours, not minutes). The ELISA analyses in Chapter 2 suggest that plasma levels of E2 in the high E2 group decreased to levels observed in the low E2 group by the time behavioural testing occured. Thus, any differences between high and low E2 rats in the LI, reversal learning, and perseveration experiments presented here can be primarily attributed to the long-term effects of E2. The methodological choice of administering E2 ~16 hours prior to testing makes these studies comparable to previous research in this lab and the field, but does not allow for an examination of the rapid effects of estrogens on these behaviours. Future studies should administer E2 30 min prior to testing to determine whether estrogens also have rapid effects on LI reversal learning and perseveration. In contrast, since the Low and High E2 replacement groups have low plasma levels of E2 from the subcutaneous capsules, differences observed between the E2 replacement groups and the No E2 replacement group are likely mediated by both the long-term and rapid effects of E2. With this experimental design it is not possible to determine whether E2-induced behavioural changes are the result of binding at nuclear ERs or mERs.

There are a limited number of studies that have specifically investigated the rapid cognitive effects of estrogens in females. Chapter 6 of this thesis describes an experiment

conducted to specifically assess the rapid effects of E2 on multiple memory systems. This study showed that microinfusions of E2 in the PFC bias female rats towards use of place memory in under 15min (Almey et al., 2014). This demonstrates mERs in to the PFC are functional, since binding at them rapidly alters navigational strategies. There are a select few other publications which also demonstrate the rapid behavioural effects of E2. Becker and colleagues have conducted numerous experiments demonstrating that E2, administered >30min prior to testing alters rotational behaviour (Becker, 1990; Schultz et al., 2009), demonstrating that rapid E2-induced changes in dopamine have behavioural consequences. Additionally, E2 administered immediately following training in an object recognition task improves memory performance when tested 4 hours later; this effect is not observed if E2 is administered 45min following training, suggesting that E2 rapidly affects memory consolidation (Inagaki et al., 2010). Additionally, systemic injections of E2 rapidly improve both social and object recognition memory, and effect that is mediated by binding at GPER1 (Ervin, Phan, Gabor, and Choleris, 2013; Gabor, Lymer, Phan, and Choleris, 2015). There is additional evidence for rapid effects of estrogens on behaviour in males, as systemic administration of E2 rapidly (15min) increases sexual behaviours (Cross and Roselli, 1999; Kaufman, Kelly, and Roselli, 2014) and aggression in males (Trainor, Finy, and Nelson, 2008). To the best of our knowledge, this represents the extent of research examining the rapid effects of estrogens on cognition.

It is clear that administration of E2 can have rapid effects on behaviour, but the vast majority of experiments examining the effects of E2 on cognition exclusively examine the long-term effects of estrogens. These long-term effects of estrogen occur through binding at nuclear ER and mERs, since binding at both types of receptors can result in changes in protein production in cells. However, there is a paucity of research examining the rapid effects of estrogens on cognition. As outlined in Chapter 1, there is a large body of research demonstrating that estrogens rapidly affect transmission in numerous regions via binding at mERs. However little research has furthered these findings by showing that these rapid estrogen-induced changes in transmission translate into changes in behaviour/cognition. Future research is needed examining the rapid behavioural effects of estrogens. Investigation into potential interactions between long-term and rapid effects of estrogens are required, since there is some evidence to suggest that there may be opposing consequences of the long-term and rapid effects of E2 (Thompson and Moss, 1994).

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