Neuropharmacological mechanisms underlying the effect of medial preoptic area dopamine in the sexual behaviour of female rats

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ABSTRACT

Neuropharmacological mechanisms underlying the effect of medial preoptic area dopamine in the sexual behaviour of female rats

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Dopamine (DA) in the medial preoptic area (mPOA) mediates the display of solicitational behaviours in the female rat. Previous work from my Masters' thesis demonstrated that a DA D1 receptor (D1R)-mediated excitatory state appears to occur in females hormonally-primed with both estradiol benzoate (EB) and progesterone (P). Conversely, a DA D2 receptor (D2R)-mediated inhibitory state appears to occur in females primed only with EB. The goals of the current thesis were to understand the mechanisms by which this control occurs, and to incorporate this understanding into the bigger framework of neural control underlying female sexual behaviour. To accomplish this, a variety of techniques were utilized. First, the same behavioural results observed previously were replicated through an endogenous alteration of DA release. It was found that administration of ascorbic acid produced an increase in solicitational, but not consummatory behaviours, in females in both hormonal conditions. Three techniques were then employed to study changes in DA receptors (DAR) in the mPOA under three hormonal profiles. Immunohistochemistry determined the number of neurons in the mPOA containing D1R or D2R, DAR protein levels were analyzed using Western blotting, and DAR functional binding levels were examined using autoradiography. The results from all three techniques supported the previous behavioural findings that EB+P females have a higher D1R/D2R ratio, and thus a D1R-mediated system, and EB-alone females have a lower D1R/D2R ratio, and thus a D2R-mediated system. Finally, fluorescence immunohistochemistry was used to map connections from the mPOA to areas known to regulate female sexual behaviour. These pathways were investigated for neuronal type and presence of DAR under different hormonal conditions. Modifications were found, demonstrating that ovarian steroids alter the mechanisms underlying solicitational behaviour via projections to other areas implicated in female sexual behaviour. A conceptual model is presented here that integrates the data in this thesis along with

other known networks that control female sexual behaviour. This model has important implications for the treatment of female sexual desire disorders, and places a central role of DA terminals in the mPOA in the regulation of motivated behaviours in general.

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"When the road is this long, you meet a lot of travellers."

- Dean Graham, right now, this thesis.

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I dedicate this thesis to Grandma Elva. I hope you are telling all your friends up there about your grandson who is now, finally, a “Doctor in Psychology”.

CONTRIBUTION OF AUTHORS

Chapter 2. Infusions of ascorbic acid into the medial preoptic area facilitate appetitive sexual behavior in the rat.

M. Dean Graham. Conceived and designed the experiment. Performed surgeries. Infused drug. Collected data. Conducted all statistics and interpreted findings. Wrote the manuscript.

James G. Pfaus. Conceived and designed the experiments. Contributed to data interpretation and writing of the manuscript.

Chapter 3. Ovarian steroids alter dopamine receptor populations in the medial preoptic area of female rats: Implications for sexual motivation, desire, and behaviour.

M. Dean Graham. Conceived and designed the experiments. Performed surgeries. Collected data in all the experiments. Conducted all statistics and interpreted findings. Wrote the manuscript.

James Gardner Gregory. Helped with data collection for Western blotting section.

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Wayne G. Brake. Helped conceive and design the autoradiography section. Helped with data collection for autoradiography section.

James G. Pfaus. Conceived and designed the experiments. Contributed to data interpretation and writing of the manuscript.

Chapter 4. Steroid hormones modulate dopamine receptor subtypes on glutamate and GABA neurons in the medial preoptic area of female rats that project to the ventromedial hypothalamus and ventral tegmental area: A triple-labelling study.

M. Dean Graham. Conceived and designed the experiments. Performed surgeries. Collected data. Conducted all statistics and interpreted findings. Wrote the manuscript.

Jason W. Payne. Helped perform surgeries. Helped with data collection.

Katuschia Germé. Conceived and designed the experiments. Helped with data collection.

James G. Pfaus. Conceived and designed the experiments. Contributed to data interpretation and writing of the manuscript.

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ABBREVIATIONS

AA	ascorbic acid
ANOVA	analysis of variance
ArcN	arcuate nucleus
cAMP	cyclic AMP
COUP	chicken ovalbumin upstream promoter
CP	caudate putamen
D1R	dopamine D1 receptor
D2R	dopamine D2 receptor
D5R	dopamine D5 receptor
DA	dopamine
DAB	diaminobenzidine
DAR	dopamine receptor
DARPP-32	dopamine and cAMP-regulated phosphoprotein-32
E2	17-β-estradiol
EB	estradiol benzoate
ER	estrogen receptor
FG	Fluoro-Gold
FIHC	fluorescent immunohistochemistry
GABA	γ-aminobutyric-acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLU	glutamate
GnRH	gonadotropin releasing hormone
GPER1	G-protein coupled estrogen receptor 1
icv	intracerebroventricular
LH	luteinizing hormone
MeA	medial amygdala
MeApd	posterodorsal medial amygdala
mER	membrane-bound estrogen receptors

mERα	membrane-associated estrogen receptor-alpha
mGluR1a	membrane glutamate receptor 1a
MOR	μ-opioid receptor
mPOA	medial preoptic area
mPR	membrane-bound progesterone receptors
MRF	medullary reticular formation
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
OT	oxytocin
OVX	ovariectomy
P	progesterone
PAG	periaqueductal gray
PAQR	progestin and adipoQ receptor
PB	phosphate buffer
PGRMC1	progesterone receptor membrane component 1
PHv	ventrocaudal posterior hypothalamus
PKA	protein kinase A
PLC	prelimbic cortex
POMC	pro-opiomelanocortin
PP-1	protein phosphatase-1
PPN	periventricular preoptic nucleus
PR	progesterone receptor
PRL	prolactin
PVH	periventricular hypothalamus
SN	substantia nigra
SUM	supramammillary nucleus
TH	tyrosine hydroxylase
THP	dihydroxyphenyl-tetrahydrothienopyridine
VCS	vaginocervical stimulation
VMH	ventromedial hypothalamus

VMHvl	ventrolateral ventromedial hypothalamus
VTA	ventral tegmental area
ZI	zona incerta

CHAPTER 1: GENERAL INTRODUCTION

Sexual behaviour in the female rat consists of three phases: appetitive, consummatory, and precopulatory (Pfaus, Kippin, & Coria-Avila, 2003). Appetitive behaviours are performed to gain access to a goal, and thus bring a female into close proximity to a male, signalling to him her interest in copulation. Copulation transpires over a series of bouts, and is able to occur because of stereotyped, sexually differentiated, and species-specific consummatory behaviours. Occurring both before and during copulation, precopulatory behaviours act as a transition from appetitive to consummatory behaviour (Pfaus et al., 2003), and these are considered similar to the proceptive behaviour originally coined by Beach (1976). This transitional behaviour from appetitive to consummatory happens through the triggering of male mounting, which in turn elicits the reflexive lordosis posture, the only unambiguous consummatory response (Pfaff & Schwartz-Giblin, 1988; Pfaus, 1999). Solicitations, hops and/or darts, and pacing behaviour are the most commonly studied precopulatory behaviours, and can be used to infer sexual desire (Pfaus, Ismail, & Coria-Avila, 2010), and as such, for the purposes of this thesis they shall all be referred to as solicitations. Solicitational behaviours are thus those that entice the male to move from distal to proximal, arousing him in order to complete the goal of copulation, increasing the female's likelihood of becoming pregnant (Erskine, Kornberg, & Cherry, 1989; Pfaus et al., 2003). As females receive a large number of intromissions during multiple copulatory series, solicitations cease, pacing increases, lordosis reflex intensities diminish, and females display more antagonistic behaviour toward males (Bermant, 1961; Erskine, 1985; Krieger, Orr, & Perper, 1976; McClintock & Adler, 1978; Peirce & Nuttall, 1961; Pfaus, Smith, Byrne, & Stephens, 2000). Therefore, while lordosis and solicitations frequently occur throughout copulatory sessions, they are mutually exclusive and cannot occur simultaneously.

Under the control of gonadal hormones (Beach, 1976; Pfaff & Schwartz-Giblin, 1988), female sexual behaviour is a stereotypical display that encompasses different processes enabling the female the move into close proximity to a male. This starts with the animal seeking out a sexual partner to engage in sexual activity. Sexual arousability, as conceived by Whalen (1966), results from a response to hormonal and neurochemical changes that signal sexual desire and arousal, culminating in an internal state commonly thought of as "sex drive" (Pfaus et al., 2003). These changes include multiple components requiring communication between different areas of the brain. One of these areas, the medial preoptic area (mPOA), has long been a controversial

area in regards to its precise role. Only recently has research started to focus on how the mPOA can control behavioural output for the display of female sexual behaviour, and evidence indicates that this control is through dopamine (DA) and its receptors (DAR) (e.g., Graham & Pfaus, 2010; Graham & Pfaus, 2012). However, how the structure of the mPOA, and DAR in particular, are altered in response to gonadal hormones has yet to be fully determined, and thus it remains unknown how changes in the female rat across the estrous cycle contribute to the timing and display of copulatory behaviour.

Hormonal Background

The full display of female sexual behaviour, consisting of all three phases mentioned above, depends on the sequential release of ovarian steroid hormones 17β-estradiol (E2) and P, and their receptors, concentrated throughout hypothalamic-limbic circuits (Beach, 1976; Pfaff & Schwartz-Giblin, 1988). Within the ventromedial nucleus of the hypothalamus (VMH), for example, E2 binding activates lordosis and P promotes precopulatory behaviours (Whalen, 1974). Ovarian steroid receptors are found within many brain areas throughout these hypothalamic circuits, including the ventral tegmental area (VTA) (Frye, 2001; Frye & Walf, 2008) and the mPOA (MacLusky & McEwen, 1978). Ovarian and plasma E2 levels gradually increase throughout the estrous cycle, peaking on the day of proestrus, while P levels are relatively high throughout, reaching maximal levels on the afternoon of proestrus. During estrous, when the females are in behavioural heat, ovarian and plasma levels of E2 and P are low (Butcher, Collins, & Fugo, 1974; Nequin, Alvarez, & Campbell, 1975; Belanger, Cusan, Caron, Barden, & Dupont, 1981). Removal of the ovaries, called an ovariectomy (OVX), eliminates the female's ability to display any sexual responses; subsequent exogenous treatment with the synthetic EB and P restores all three phases 48 hours and 4 hours, respectively, after treatment (Pfaff, 1980; Feder, 1984).

Estrogen can quickly change neurophysiology through actions involving membrane receptor signalling. For example, rapid effects of E2 are observed in the striatum (see below), an area where minimal classical nuclear ERs are found (Pfaff & Keiner, 1973; Shughrue, Lane, & Merchenthaler, 1997). These effects may instead be caused by a recently identified non-classical G protein-coupled estrogen receptor 1 (GPER1, formerly referred to as GPR30), a type of 7-transmembrane G protein-coupled receptor (GPCR), which are characterized by mediating rapid changes at the level of second messengers and by regulating kinase pathways (Luttrell, 2006).

Contrary to typical GPCRs, GPER1 is mostly expressed intracellularly in the endoplasmic reticulum, with only a small percentage found on the cell surface, meaning it may also be activated intracellularly by E2, which diffuses across cell membranes (Revankar, Mitchell, Field, Burai, Corona, Ramesh, Sklar, Arterburn, & Prossnitz, 2007). Therefore, in response to E2, GPER1 can have either transcriptional or rapid actions (Prossnitz & Barton, 2009). GPER1 is found in moderate amounts in the striatum, including the caudate putamen (CP) (Hazell, Yao, Roper, Prossnitz, O'Carroll, & Lolait, 2009; Prossnitz & Barton, 2009), and in intense amounts in the mPOA (Hazell et al., 2009).

In addition to the traditional genomic signal transduction of classic nuclear ERs, non-genomic signalling has been observed, characterized by rapid response using membrane-initiated estradiol signalling. Multiple proteins have been suggested as having this function, including ERα, ERβ, and GPER1 (for a review, see Mermelstein & Micevych, 2008; Micevych & Dewing, 2011). Although the role of these membrane ERs (mER) in female sexual behaviour remains unsettled, data suggests a role for them in response to E2. For example, Dewing and colleagues (2007) used a form of E2 impermeable to the cell membrane, and observed that lordosis was increased through stimulation of mERα and an interaction with metabotropic glutamate receptors in the mPOA. Furthermore, the role of mER in conjunction with genomic ERs has been examined, determining that membrane actions facilitate the effects of ERs on lordosis, with signalling pathways involving PKA and PKC mediating the effect (Kow & Pfaff, 2004). This indicates that E2 can act on mER to either substitute or potentiate the effects of classical ERs on female sexual behaviour.

Similar to E2, P is important for controlling the release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH), and for enhancing female sexual behaviour. It also is involved in several neural processes such as neurogenesis, neuroinflammation, neuroprotection, neuronal cell death, cognitive function, and emotion (for review, see Thomas & Pang, 2012; Petersen, Intlekofer, Moura-Conlon, Brewer, Del Pino Sans, & Lopez, 2013a). Progesterone receptors (PR) has been shown to be induced by E2 both naturally (MacLusky & McEwen, 1978) as well as following exogenous EB priming (Parsons, McGinnis, & McEwen, 1981). These E2-induced PR were then shown to be implicated in female sexual responsiveness, where the increase in PR, in response to E2, creates a more behaviourally responsive female rat (Moguilewsky & Raynaud, 1979; Parsons, MacLusky, Krey, Pfaff, & McEwen, 1980; Parsons et

al., 1981; Parsons, McEwen, & Pfaff, 1982) and guinea pig (Blaustein & Feder, 1979a; Blaustein & Feder, 1979b). High levels of ER have been found in many areas associated with female sexual behaviour, including the mPOA and the VMH (Rainbow, Parsons, MacLusky, & McEwen, 1982), and these areas have also been demonstrated to show high levels of E2-induced PR (Parsons, Rainbow, MacLusky, & McEwen, 1982).

Progesterone can also induce rapid changes in neurophysiology. Three distinct mechanisms allow for PR activation in the brain: classical/genomic, non-classical/non-genomic, and ligand-independent. The first two mechanisms are ligand-dependent, indicating they require activation by P binding. This activation can occur through cell surface progestin binding receptors, in the classical nuclear hormone receptor sense, requiring the occupation and activation of PR by P (Tsai & O'Malley, 1994). The non-classical mechanism occurs through coupling with second messenger signalling cascades using pathways independent of transcriptional regulation at the genomic level. These can be cell membrane or cytoplasmic signal transduction pathways (for review, see Mani, 2006). The third mechanism, ligand-independent, occurs through PR activation by factors other than P (Mani & O'Malley, 2002). It has been suggested that these three methods of activation are in fact not mutually exclusive, but achieve their behavioural endpoints through interaction and/or synergy with each other (for review, see Mani, 2003; Mani & Blaustein, 2012).

Two groups of non-classical PR are known. One consists of membrane PR (mPR) and are part of the progestin and adipoQ receptor (PAQR) family, and are thought to act through cAMP (Thomas & Pang, 2012), however they are not always found coupled to G proteins or found in the plasma membrane, nor are most types found in neural tissue (as reviewed by Petersen et al., 2013a; Petersen et al., 2013b). Unlike mPR, the second group is found in neural tissue, and contains a cytochrome b5-heme/steroid binding domain (Kimura, Nakayama, Konishi, Terasawa, Ohta, Itoh, & Fujimoto, 2012). This group includes progesterone receptor membrane component 1 (PGRMC1), which is found in high levels in many areas important for sexual behaviour, such as the mPOA, VMH, and VTA (Krebs, Jarvis, Chan, Lydon, Ogawa, & Pfaff, 2000; Intlekofer & Petersen, 2011).

Sexual Behaviour in the Ventromedial Nucleus of the Hypothalamus

Ovarian hormones are responsible for displaying lordosis at appropriate times, affecting neurochemical pathways among other neurobiological changes. The VMH is the main site of

action for these hormones in controlling lordosis, and as such is the most widely studied area. Studies have consistently demonstrated that the VMH plays a critical role in this display (Mathews & Edwards, 1977; Pfaff & Sakuma, 1979a; Pfaff & Sakuma, 1979b; Calizo & Flanagan-Cato, 2002; Flanagan-Cato, 2011). For example, lordosis is inhibited following bilateral electrolytic lesions (Pfaff, 1980) and knife transections (Mathews & Edwards, 1977; Pfaff & Sakuma, 1979b; Pfeifle, Shivers, & Edwards, 1980) of this area, and an enhancement of lordosis is observed following electrical stimulation (Pfaff & Sakuma, 1979a). In the VMH, estrogen activation facilitates lordosis (e.g., Mathews & Edwards, 1977; Rubin & Barfield, 1983), and supplemental evidence demonstrates that progesterone increases this behaviour when administered to this area as well (DeBold & Malsbury, 1989; Pleim, Baumann, & Barfield, 1991; Frye & Vongher, 1999).

Within the VMH, the effects of glutamate on lordosis have been widely studied, the results of which show that it plays an inhibitory role. Lordosis is decreased following infusions of glutamate itself, or any of its agonists (i.e., kainic acid, AMPA, and NMDA) (Kow, Harlan, Shivers, & Pfaff, 1985; McCarthy, Curran, & Feder, 1991; Georgescu & Pfaus, 2006a). Glutamate antagonists (i.e., AP-5, CNQX, and DNQX) have similarly been found to increase lordosis (Georgescu & Pfaus, 2006b). Ovarian hormones have an effect on this neurotransmitter within the VMH (e.g., Kow et al., 1985; Schumacher, Coirini, & McEwen, 1989; McCarthy, 1995), as HPLC studies indicate glutamate levels increase following estradiol treatment (Luine, Grattan, & Selmanoff, 1997; Luine, Wu, Hoffman, & Renner, 1999), and this effect is reversed following subsequent progesterone administration (Luine et al., 1999). It should be noted that it has also been found using microdialysis that EB treatment, with or without P, decreases glutamate release (Georgescu, Afonso, Graham, & Pfaus, 2014), and glutamate neuron activation also has been shown to decrease following a similar regimen (Georgescu, Sabongui, Del Corpo, Marsan, & Pfaus, 2009).

Dopamine is another neurotransmitter found in the VMH that affects lordosis. It is increased following infusions of DA agonists to the VMH (Foreman & Moss, 1979; Apostolakis, Garai, Fox, Smith, Watson, Clark, & O'Malley, 1996). Similarly, infusions of DA antagonists decrease lordosis (Foreman & Moss, 1979). Dopamine's effect within the VMH on lordosis has been narrowed down to being driven by the DA D1 receptor (D1R) subtype, and more specifically the D5 receptor (D5R) (Apostolakis et al., 1996). Mani and colleagues have

extended these findings to show that this stimulatory effect might also be due to DA D1R activation in the VMH, resulting in the activation of PRs (e.g., Mani, Allen, Clark, Blaustein, & O'Malley, 1994; Mani, Allen, Lydon, Mulac-Jericevic, Blaustein, DeMayo, Conneely, & O'Malley, 1996; Mani, Fienberg, O'Callaghan, Snyder, Allen, Dash, Moore, Bibb, Greengard, & O'Malley, 2000; Mani, Mitchell, & O'Malley, 2001).

The mPOA and Male Sexual Behaviour

Other regions are responsible for the control of sexual behaviour, one of which is the mPOA. This area, located anterior to the hypothalamus, is integral in a wide variety of physiological and behavioural functions. These motivated behaviours include feeding (e.g., Patterson, Murphy, Thompson, Smith, Meeran, Ghatei, & Bloom, 2006; Leibowitz, Akabayashi, Wang, Alexander, Dourmashkin, & Chang, 2007), sodium and fluid balance (e.g., Swanson, Kucharczyk, & Mogenson, 1978; Swanson & Mogenson, 1981; Bourque, Oliet, & Richard, 1994), thermoregulation (e.g., Squires & Jacobsen, 1968; Bicego, Barros, & Branco, 2007; Kumar, Vetrivelan, & Mallick, 2007), arousal (e.g., Kumar et al., 2007; Szymusiak, Gvilia, & McGinty, 2007), gonadotropin release (e.g., Gorski, 1968; Wiegand & Terasawa, 1982; Funabashi, Mitsushima, Nakamura, Uemura, Hirahara, Shinohara, Suyama, & Kimura, 2002; Mahesh & Brann, 2005), and maternal motivation and behaviour (e.g., Numan, 1974; Jacobsen, Terkel, Gorski, & Sawyer, 1980; Numan & Insel, 2003; Lonstein & Morrell, 2007).

Of particular importance, the mPOA is critical for the display of sexual behaviour, although what is largely known has been determined in males (as reviewed by Dominguez & Hull, 2005; Hull, Wood, & McKenna, 2006; Hull & Dominguez, 2007). Efferent projections from the mPOA are responsible for initiating copulation in male rats (Hull, Lorrain, Du, Matuszewich, Lumley, Putnam, & Moses, 1999), and males who have lesions to the mPOA are unable to begin mounting, and do not display thrust patterns, though they still may demonstrate appetitive behaviours to be with a female (Hansen, Kohler, Goldstein, & Steinbusch, 1982; Everitt, 1990). The major efferents from the mPOA are to hypothalamic, midbrain, and brain stem nuclei that regulate autonomic and somatomotor patterns, motivational states, mediate motor activity, and are implicated in reward (Fahrbach, Morrell, & Pfaff, 1986; Simerly & Swanson, 1988; Sinnamon, 1992). In addition to these efferents, the mPOA also contains a high concentration of ovarian steroid receptors (Pfaff & Keiner, 1973; Parsons et al., 1982a), and

other receptors, such as dopaminergic, that allow for the influence of sensory processing in regards to sexually relevant stimuli (Hull et al., 1999), to be discussed below.

The mPOA can be thought of then as being important for the preparatory behaviour in regards to sex drive for the male. Specifically, the mPOA gets the body prepared for sex, as ablation studies demonstrate that impairment to this area results in an abolishment of male sexual behaviour in every species studied to date (Hull, Meisel, & Sachs, 2002). This is further confirmed by stimulation studies, as electrical stimulation of the mPOA promotes erection (Giuliano, Bernabe, Brown, Droupy, Benoit, & Rampin, 1997) and the urethrogenital reflex (Marson & McKenna, 1994), and also reduces the postejaculatory interval, the amount of time required for an ejaculation to be produced, and the number of intromissions performed before ejaculation (Malsbury, 1971; Rodriguez-Manzo, Pellicer, Larsson, & Fernandez-Guasti, 2000). For males that have reached sexual satiety, stimulation of the mPOA does not restart copulation, indicating that this aspect of male sexual behaviour is not under the control of the mPOA (Rodriguez-Manzo et al., 2000).

Sexual behaviours in the rodent are stereotypical in display, occurring only when the timing to do so is correct. This requires that the animal be in the proper frame of mind corresponding to the ideal reproductive state. For the male, this means that a penile erection is produced at the time when the male is able to mount a sexually receptive female. Research indicates that the mPOA is important in integrating information so that this coordination occurs. Different cells located in the mPOA are activated during stages of male sexual behaviour, as some show increased activity preceding copulation, corresponding to appetitive aspects of sexual behaviour, while others are activated only during copulation (Shimura, Yamamoto, & Shimokochi, 1994). For example, odor from a receptive female also increases cellular activation in the mPOA (Bressler & Baum, 1996; Kelliher, Liu, Baum, & Sachs, 1999), and noncontact erections also increase activity in this area (Kelliher et al., 1999). This indicates that the mPOA helps prepare the male for copulation by the promotion of erection. Furthermore, the amount of activity in the mPOA of a variety of rodents is increased in relation to increasing amount of copulation (Robertson, Pfaus, Atkinson, Matsumura, Phillips, & Fibiger, 1991; Baum & Everitt, 1992; Heeb & Yahr, 1996; Kollack-Walker & Newman, 1997; Veening & Coolen, 1998). Similar evidence exists for monkeys, while also demonstrating that the role of the mPOA ceases after ejaculation (Oomura, Aou, Koyama, Fujita, & Yoshimatsu, 1988). The combination of

these studies suggests that the mPOA acts as an area of integration, an important role in regulating copulation. This is because certain sexual behaviours are mutually exclusive, and thus can only be displayed individually. The implication being that for males, ejaculation cannot be reached unless an erection is sustained first, and the mPOA is responsible for this timing and ordering of these behaviours.

The mPOA and Female Sexual Behaviour

Historically, the mPOA has been reduced to being the predominant area responsible for the control of male sexual behaviour, and the VMH being the predominant area in the control of female sexual behaviour. For females, this has been based on the majority of the literature focusing on consummatory behaviours, as findings demonstrate that VMH lesions inhibit lordosis (Mathews & Edwards, 1977; Pfaff & Sakuma, 1979b; Pfaff, 1980), while electrical stimulation facilitates it (Pfaff & Sakuma, 1979a). The VMH shares connections to the mPOA (Conrad & Pfaff, 1975), although the nature of these connections is unknown. In regards to male sexual behaviour, efferent projections from the mPOA are necessary for copulation to be initiated (Hull et al., 1999), as males with mPOA lesions are unable to start mount and thrust patterns, although appetitive behaviour towards a receptive female is still noticed (Hansen et al., 1982; Everitt, 1990). For females, the mPOA has shown the opposite effects in regards to lordosis, with early studies finding enhancements following lesions to this area (Law & Meagher, 1958; Powers & Valenstein, 1972; Clemens, Smalstig, & Sawyer, 1976; Takeo, Chiba, & Sakuma, 1993) and decreases following electrical stimulation (Napoli, Powers & Valenstein, 1972; Moss, Paloutzian, & Law, 1974; Pfaff & Sakuma, 1979a; Takeo et al., 1993). This inhibitory influence on lordosis could potentially be through direct outputs to the VMH (Chiba & Murata, 1985). It should be noted however that many other studies found results inconsistent with this premise (e.g., Singer, 1968; Numan, 1974; Gray, Soderstein, Tallentire, & Davidson, 1978; Bast, Hunts, Renner, Morris, & Quadagno, 1987). This gave rise to the idea that the mPOA plays very different roles for the control of sexual behaviour between the two sexes, being the principle area in the control of male displays, but having either no effect, or an inhibitory role, on lordosis.

However, female sexual behaviour is not just lordosis, and the role of the mPOA in regards to female sexual behaviour was later thought to be context-specific, depending on the environment used to test sexual behaviour (Erskine, 1989). Whitney (1986) found that if the

chamber was small and the female unable to escape the male, mPOA lesions did facilitate lordosis, but no effect on solicitation behaviour was observed. If however the female was tested in an exit test, done with a chamber that allowed her to escape the male at her preferred interval, mPOA lesions had no effect on lordosis, although solicitational behaviour, as measured through pose frequency, was reduced. As well, rats with mPOA lesions spent less time with males, and thus received fewer mounts, intromissions, and ejaculations (Whitney, 1986). Thus, previous discrepancies in the literature may be due to the female's inability to perform proper solicitational behaviour because of the constraints of the testing environment. This would lead the female to show changes in lordosis behaviour to trigger male mounting rather than the typically displayed solicitations. This research suggests that the mPOA may control motivational aspects of female sexual behaviour through the management of solicitations instead of consummatory behaviours, which would fall under the control of the VMH predominantly.

Evidence for the viewpoint that the mPOA helps regulate solicitational behaviour originally comes from an important finding by Hoshina and colleagues (1994). They discovered that excitotoxic lesions to the mPOA produced not only an increase in lordosis, but a large reduction in solicitations. A similar study performed by Guarraci and colleagues (2004) found a comparable decrease in solicitations following mPOA lesions, although this study did not find an alteration in lordosis. These data are consistent with the idea that the mPOA is important for the expression of solicitational behaviour, whereas its role in lordosis, if any, may depend on the sexual context. Thus, the mPOA may be responsible for the control of sexual motivation through the management of solicitations, while the VMH controls consummatory behaviour. In this way, although solicitations and lordosis are mutually exclusive, both may occur in a synchronized manner, as a consequence of activational "leakage" across multiple sets of receptors and/or brain areas. This would result in an integration of sexual behaviours, where one set is being displayed while the other set is inhibited.

The integration of sexual behaviours could exist through communication between the mPOA and the VMH, as previously mentioned (Chiba & Murata, 1985). Neuronal activation in the mPOA has been shown to occur in response to mechanical stimulation of the cervix (Haskins & Moss, 1983), vaginocervical stimulation (VCS) from a male partner during copulation (Pfaus, Kleopoulos, Mobbs, Gibbs, & Pfaff, 1993), and VCS via a glass rod, an artificial laboratory manipulation which mimics a male (Pfaus, Marcangione, Smith, Manitt, & Abillamaa, 1996).

This indicates that the receipt of sexual stimuli from the male via the cervix and/or genitals causes activation in the mPOA, which may be important for the animal to know to disengage from the lordosis reflex and prepare for a runaway. Evidence supporting this comes from a finding that lesioning the mPOA increases the latency for females to return to the male following intromissions and ejaculations only, with latencies following mounts unaffected (Yang & Clemens, 2000). This is interesting in that it demonstrates that mPOA lesions influence the temporal pattern of female sexual behaviour only when VCS is involved, leading the authors to suggest that the mPOA plays an important role in processing and responding to VCS, which is in agreement with the idea that the mPOA is important for the timing of female sexual responses. Furthermore, lesioned animals also spent less time with the male, lending additional support that the mPOA also is important in sexual motivation (Yang & Clemens, 2000).

An explanation regarding some of the confusing and contradictory findings of the mPOA in terms of female sexual behaviour may come from an important study performed by Kato and Sakuma (2000). In it, the authors identified four different types of neurons in this area, asserting that the mPOA may have the ability to preferentially respond to varying aspects of both solicitational and consummatory behaviours. Type 1 neurons signify the motivational state of the rat, represented by solicitations, as they increased their firing rate when these behaviours were initiated by the female, continuing until the male intromitted, at which time the firing was suppressed. Type 2 and Type 3 neurons may fire in response to visceral or somatosensory input, as they showed activation when the female was mounted and intromitted by the male, respectively. Type 4 neurons may be responsible for inhibiting lordosis, as inhibition of these neurons occurred immediately before and during any display of lordosis by the female (Kato & Sakuma, 2000). It then stands to reason that the mPOA may have neurons that can respond to all types of sexual behaviour, and these responses are critical in the organization, integration, and timing of the wide behavioural repertoire displayed by the female throughout the copulatory session.

Since female sexual behaviour consists of both appetitive and consummatory behaviours throughout several copulatory bouts, the mPOA may be much more complex in its role, one of which may be to time. Since rats cannot take a stationary lordosis posture and perform running solicitations simultaneously, the mPOA may play a role in toggling those two behaviours in time. Thus, the mPOA may stimulate appetitive responses and somatomotor patterns that are dynamic,

which may ultimately involve the activation of mesolimbic dopamine at the expense of behaviours that are stationary, like lordosis. The mPOA may play a role in mediating that toggle, and when the VMH is inhibited, the mPOA may in fact be activated, and vice versa, so that these two brain areas exist in a reciprocal relationship.

Sexual Reward and Dopamine

An essential component of sexual behaviour is that of sexual desire. Sexual desire is expressed by goal-directed sexual behaviours, centred on receiving positive sexual reinforcement. This sexual reward is related to the sexual desire it evokes, so that the strength of the sexual desire is typically controlled by the reward expectation. To that end, sexual desire is thought to comprise the strength of the reward the individual seeks, the excitement that precedes its receipt, and the work necessary to obtain it (Pfaus, 2009; Pfaus, Wilkins, Dipietro, Benibgui, Toledano, Rowe, & Couch, 2010). Sexual rewards come in two types, primary and secondary reinforcers. For female rats, primary reinforcement is seen in the ability to pace copulation, while secondary cues are stimuli associated with sexual gratification. Examples often used are odors or place cues (for a review, see Pfaus, Kippin, & Centeno, 2001). As with other types of reward, DA appears to play a vital role in both attending to these sexual incentives, and modulating appetitive responses towards them.

Dopamine is a catecholamine neurotransmitter linked to a wide array of reward-related appetitive behaviours (e.g., Wise & Rompre, 1989; Salamone, 1994; Ikemoto, Glazier, Murphy, & McBride, 1997; Sutton & Beninger, 1999). There are two functionally distinct components of DA release: phasic and tonic. Phasic release, caused by action potentials and thought to be responsible for most behavioural observances, results in pulses of DA which is then removed quickly by reuptake mechanisms (Grace, 1991; reviewed in Grace, 2002). Tonic release results in a low concentration of DA, which can act extrasynaptically to cause feedback inhibition of phasic DA release. A prevalent characteristic of DAergic action is augmentation of sensorimotor function, thought to be achieved through removal of tonic inhibition (Chevalier & Deniau, 1990). Removal of this inhibition permits other steroid hormones to increase the responsiveness of specific neurons, meaning that DA may not directly influence behaviour on its own, but instead by causing stimuli to have easier access to hormonally-primed output pathways (Hull, Du, Lorrain, & Matuszewich, 1997).

Five DAR have been identified, starting with DA D1R and DA D2R (Kebabian & Calne, 1979; Stoof & Kebabian, 1984), followed by DA D3R (Sokoloff, Giros, Martres, Bouthenet, & Schwartz, 1990), DA D4R (Van Tol, Bunzow, Guan, Sunahara, Seeman, Niznik, & Civelli, 1991), and DA D5R (Sunahara, Guan, O'Dowd, Seeman, Laurier, Ng, George, Torchia, Van Tol, & Niznik, 1991). These are G protein-coupled receptors, categorized as two subtypes: the first, known as the DA D1R-like subtype, consists of DA D1R and DA D5R; the second, collectively known as the DA D2R-like subtype, consists of the DA D2R, DA D3R, and DA D4R. The two subtypes are characterized by how they interact with adenylyl cyclase (AC): The DA D1R-like subtype stimulate AC, whereas the DA D2R-like subtype either inhibit, or have no effect on the enzyme (reviewed in Civelli, Bunzow, & Grandy, 1993; D'Souza, 2010). Most often these two receptor subtypes act synergistically, though occasionally they produce opposing influences (reviewed in Arnt, 1987).

The DA D2R has two main variants, DA D2Rshort and DA D2Rlong, differing by a 29-amino acid segment missing in the short version (O'Dowd, Nguyen, Tirpak, Jarvie, Israel, Seeman, & Niznik, 1990). Although the long form is more commonly expressed in the brain (e.g., Kukstas, Domec, Bascles, Bonnet, Verrier, Israel, & Vincent, 1991; Snyder, Roberts, & Sealfon, 1991), DA agonists and antagonists have a similar affinity for both versions (Leyson, Gommeren, Mertens, Luyten, Pauwels, Ewert, & Seeburg, 1993). Autoreceptors that are responsible for the tonic down-regulation of DA neuron activity are primarily DA D2R (Mercuri, Saiardi, Bonci, Picetti, Calabresi, Bernardi, & Borrelli, 1997).

When DA signals through DA D1R, 3'-5'-cyclic adenosine monophosphate (cAMP) increases due to activation of AC, stimulating cAMP-dependent protein kinase A (PKA). PKA in turn phosphorylates dopamine and cAMP-regulated phosphoprotein-32 (DARPP-32) at Threonine-34, which inhibits the activity of protein phosphatase-1 (PP-1) when phosphorylated. By inhibiting PP-1, many substrate proteins may be phosphorylated, including glutamate and γ-aminobutyric-acid (GABA) receptors, among others. These phosphorylated substrate proteins then induce physiological responses. Stimulation of DA D2R can attenuate DA D1R activation of AC, or decrease the phosphorylated state of DARPP-32 via calcium stimulation of protein phosphatase 2B (Greengard, Allen, & Nairn, 1999). Thus, there is an interdependence on the activity levels and uptake of DA, its neurons, and receptors. This helps determine how DAergic

activity is modulated by phasic and tonic release, a delicate balance that has been proposed to underlie normal and dysfunctional DA regulation (for a review, see Grace, 2002).

Several DA systems have been identified, with the mesolimbic system receiving a vast amount of attention. Originating in the VTA, this system is known to be critical for appetitive behaviour and reinforcement (as reviewed by Fields, Hjelmstad, Margolis, & Nicola, 2007). It is activated when a variety of motivated behaviours are performed, including many that also cause mPOA activation, such as feeding, drinking, and sexual behaviour. For example, the nucleus accumbens, an area receiving major output from the VTA, shows increased extracellular DA following copulation or when a receptive female is presented behind a barrier (Damsma, Pfaus, Wenkstern, Phillips, & Fibiger, 1992), while a non-receptive female does not elicit an increase (Wenkstern, Pfaus, & Fibiger, 1993), demonstrating that this increase is specific to a rewarding stimulus.

The mPOA and Male Sexual Reward

The mPOA is connected to this sexual reward system, sending efferents to the VTA and other areas of the mesolimbic system (Simerly & Swanson, 1988; Zahm, Cheng, Lee, Ghobadi, Schwartz, Geisler, Parsely, Gruber, & Veh, 2011), and serves to stimulate DA neurons, which account for approximately 65% of all neurons in the VTA (Margolis, Lock, Hjelmstad, & Fields, 2006; Nair-Roberts, Chatelain-Badie, Benson, White-Cooper, Bolam, & Ungless, 2008). Recently, it has been demonstrated that the mPOA modulates DA release within the VTA following cocaine administration, and that the mPOA is integral for the processing of cocaine-induced responses in both males (Will, Martz, & Dominguez, 2016), and females (Tobiansky, Roma, Hattori, Will, Nutsch, & Dominguez, 2013; Tobiansky, Will, Lominac, Turner, Hattori, Krishnan, Martz, Nutsch, & Dominguez, 2016). Interestingly, it appears that this inhibitory mPOA-VTA projection is sensitive to estradiol (Tobiansky et al., 2016).

An mPOA-VTA connection has long been included in models corresponding to areas responsible for the display of male sexual behaviour (e.g., van Furth, Wolterink, & van Ree, 1995), though this projection has been neglected when it comes to female sexual behaviour (e.g., Young & Wang, 2004). Based on extensive studies, Hull and colleagues (1995; 1999) have devised a model representing the role of DA in three integrative systems for the male rat (see Figure 1). Under this model, the mPOA focuses the motivation of the male on sexually relevant

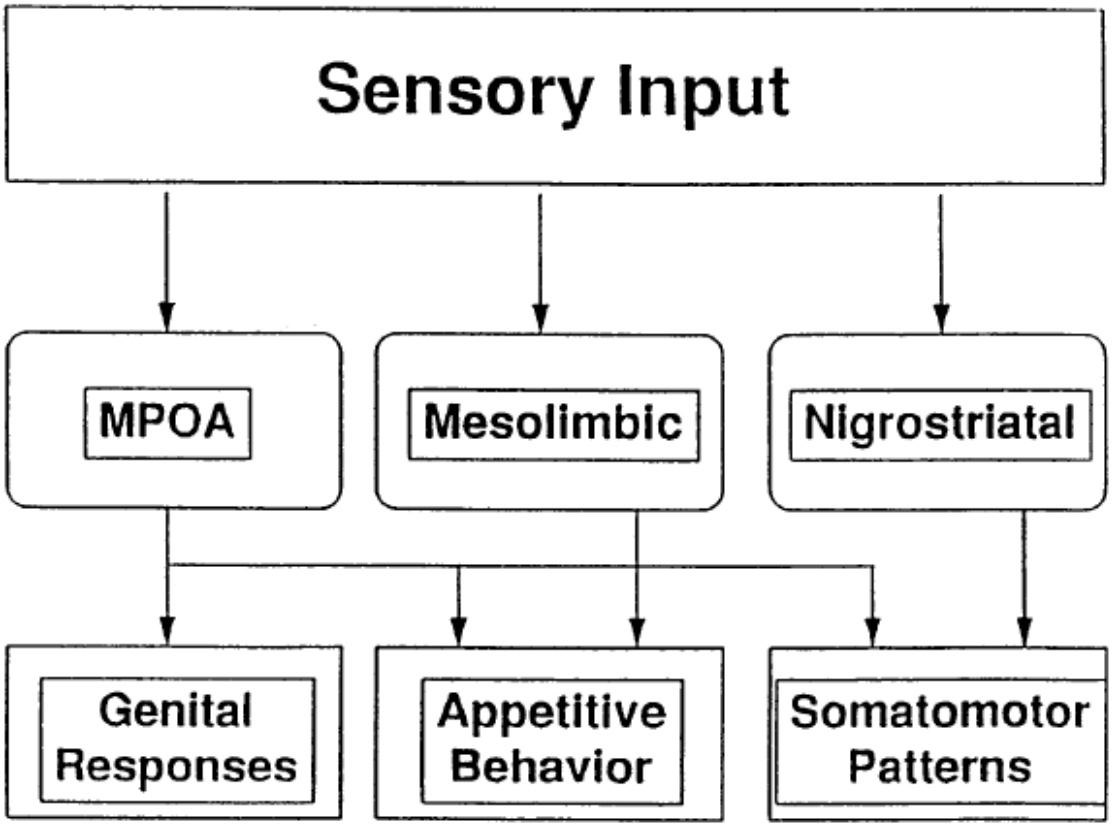

Figure 1. A conceptual model from Hull and colleagues (1999) describing how dopamine systems may regulate sexual behaviour. According to this model, dopamine in the mPOA controls genital responses, appetitive behaviour, and somatomotor patterns in response to sensory stimuli from a sexual partner. (Reprinted from Hull, Lorrain, Du, Matuszewich, Lumley, Putnam, & Moses, 1999, with permission from Elsevier).

stimuli, while simultaneously coordinating genital reflexes and producing the necessary motor patterns to perform copulation typical of the species.

Older research initially indicated that the mPOA was not important for male sexual motivation, and that it was the ventral striatal system instead (Everitt, 1990). More recent research, however, indicates that the mPOA contributes to sexual motivation to some degree in males. Lesioning the mPOA results in the male rat decreasing his pursuit of a female (Paredes, Highland, & Karam, 1993) and reduces both male rat and ferret preferences for a female partner (Edwards & Einhorn, 1986; Paredes & Baum, 1995; Kindon, Baum, & Paredes, 1996; Edwards, Walter, & Liang, 1996; Paredes, Tzschentke, & Nakach, 1998). Marmoset precopulatory behaviour is also inhibited following mPOA lesions (Lloyd & Dixson, 1988). Conflicting evidence exists, however, as non-contact erections are not affected by mPOA lesions (Liu, Salamone, & Sachs, 1997), and males with this ablation still appear to have sexual motivation as they continue to chase receptive females, investigating their anogenital region (Heimer & Larsson, 1967; Hansen & Hagelsrum, 1984). Research on mPOA lesions in other animals further confounds this understanding, as monkeys show no change in masturbation frequency although copulation has been attenuated, or even eliminated (Slimp, Hart, & Goy, 1978), cats and dogs show similar levels of sexual interest (Hart, Haugen, & Peterson, 1973; Hart, 1974), and lesioned male mice also continue to emit ultrasonic "courtship" vocalizations even though mating has been eliminated (Bean, Nunez, & Conner, 1981).

Dopamine in the mPOA

The mPOA is an integrator of DAergic inputs, receiving afferents from a wide variety of areas. For this reason, specific sources of input remain unclear. The largest input was originally thought to come from the zona incerta (ZI; Björklund, Lindvall, & Nobin, 1975; Simerly & Swanson, 1986; Wagner, Eaton, Moore, & Lookingland, 1995), a subthalamic nucleus posterior to the hypothalamus, with the ZI receiving input from the cerebellum and motor cortex (Björklund et al., 1975). These areas collectively make up the incertohypothalamic DA system, originating in the A13 cell group of the hypothalamus (Björklund et al., 1975; Wagner et al., 1995). The mPOA does receive inputs of varying strength from multiple sites that are abundant in DA neurons, including the periventricular hypothalamus (PVH) and the VTA (Chiba & Murata, 1985; Simerly & Swanson, 1986; Wagner et al., 1995), however the only study to date to anatomically trace DA projections to the mPOA was performed on postpartum females (Miller

& Lonstein, 2009). Their findings indicate that the average number of DA neurons projecting to one mPOA hemisphere is 89±16, widely distributed throughout the forebrain and midbrain (Miller & Lonstein, 2009). These areas included the ventrocaudal posterior hypothalamus (PHv; 26% of total input), the supramammillary nucleus (SUM; 13%), the VTA (8%), the substantia nigra (SN; 7%), and the periaqueductal gray (PAG; 6%).

Collectively, the results from the Miller and Lonstein (2009) study indicate that the DA A10 system is responsible for the largest portion of DAergic input to the mPOA, approximately a third of all DAergic projections. This is much larger than previously believed, though prior studies have found changes in DA content in the mPOA after A10 system manipulations (Kizer, Palkovits, & Brownstein, 1976; Lookingland & Moore, 1984). It is speculated that the inputs from the PHv and SUM may be responsible for male sexual behaviour (Miller & Lonstein, 2009), though at this time this has not been confirmed. Of the remaining DAergic input to the mPOA, very little of it was found to come from the DA A13 or A14 systems (Miller & Lonstein, 2009). These were initially thought to be the two main inputs (e.g., Björklund et al., 1975; Wagner et al., 1995), even though it was previously known that there are sparse ZI projections to the mPOA (Simerly & Swanson, 1986; Wagner et al., 1995). Miller and Lonstein (2009) explain that these original hypotheses may have been based on initial research that emphasized how DA systems within the hypothalamus were self-contained (e.g., Björklund et al., 1975). In fact, over half of the DAergic input falls outside of the DA A8-A15 groups, and taking in the approximately 33% of the DA A10 group, this leaves collectively about 17% of the remaining DA groups (Miller & Lonstein, 2009). This demonstrates that DAergic input has a widespread distribution, and at this time no individual DA group can be considered responsible for any of the behaviours DA in the mPOA is known to affect.

In addition to the role of the mPOA in modifying DA release in the VTA, a plethora of evidence suggests that DA in the mPOA itself plays an important role in the direct control of sexual motivation in the male. The presence of an estrous female causes DA release in the mPOA, which is increased further when sexual contact and copulation occur (Pfaus, Damsma, Nomikos, Wenkstern, Blaha, Phillips, & Fibiger, 1990; Hull, Eaton, Moses, & Lorrain, 1993; Hull, Du, Lorrain, & Matuszewich, 1995; Dominguez, Riolo, Xu, & Hull, 2001; Putnam, Du, Sato, & Hull, 2001; Putnam, Sato, & Hull, 2003). Conversely lesioning the mPOA, or administering DAR antagonists, affects sexual motivation (Paredes et al., 1993; Moses, Loucks,

Watson, Matuszewich, & Hull, 1995). The study of hamsters has demonstrated that chemosensory cues from the female are required for the DA release observed in the mPOA in response to mating, as those who received bilateral bulbectomies did not show this response (Triemestra, Nagatani, & Wood, 2005). In addition, although cellular activity is found in the mPOA in response to female odours before puberty is reached (Romeo, Parfitt, Richardson, & Sisk, 1998), an increase in DA activity in response to these odours is only observed once the male has reached puberty, indicating he is able to copulate (Schulz, Richardson, Romeo, Morris, Lookingland, & Sisk, 2003).

Infusion of haloperidol, a DA antagonist, to the mPOA decreases anticipatory level changes using a bilevel chamber (Pfaus & Phillips, 1991). This behaviour may reflect a male's search for a female. Similarly, infusion of another DA antagonist, flupenthixol, to the mPOA reduced the percentage of trials a receptive female was chosen by a male on an X-maze. This reduction was not accompanied by a change in running speed, indicating that a change in sexual motivation, and not motor activity, was affected by the alteration of DA activity in the mPOA (Warner, Thompson, Markowski, Loucks, Bazzett, Eaton, & Hull, 1991). Similar findings were found following infusions of the DA D1 receptor (D1R) antagonist SCH-23390 and the DA D2 receptor (D2R) antagonist raclopride. Conversely, infusion of the DA D2R agonist quinelorane increased the latency to reach the female's chamber (Moses et al., 1995). These findings appear to be specific to sexual motivation, as other general motivational states did not seem to be affected, as locomotion, eating, or drinking were unchanged. This indicates that DAergic activity in the mPOA of the male is associated with a male's ability and desire to copulate in response to a sexual stimulus.

The ability to determine the precise role of specific DA receptors on male sexual behaviour in the mPOA has proven to be more complex than the neurotransmitter itself. Predominantly based on the work done by Hull and colleagues, the role of DAR in the mPOA has been found to be critical in facilitating both erection and ejaculation, and just as importantly the transition between them. To this end, the DAR subtypes play opposing, but synergistic roles in affecting male copulation. For example, administration of the DA D2R agonist quinelorane to the mPOA increases the latency for the male to first mount and intromit the female, thus impairing copulation onset, while decreasing the latency to ejaculation by reducing the number of intromissions required (Hull, Warner, Bazzett, Eaton, Thompson, & Scaletta, 1989), and

decreasing the latency for to display ex copula genital reflexes (Bazzett, Eaton, Thompson, Markowski, Lumley, & Hull, 1991). Meanwhile, stimulation of DA D1R using the agonist dihydroxyphenyl-tetrahydrothienopyridine (THP) facilitates ex copula erections, but inhibits ejaculations (Hull, Eaton, Markowski, Moses, Lumley, & Loucks, 1992). It has now been surmised that DAR in the mPOA have different effects in the autonomic control of genital reflexes, with distinct thresholds of activation. Specifically the idea has been put forth that DA D1R stimulation promotes erection, while decreasing the latency to ejaculate through the promotion of sexual motivation by DA D2R in a synergistic manner (Markowski, Eaton, Lumley, Moses, & Hull, 1994; Moses et al., 1995). This led Hull and colleagues to conclude that DA D2R stimulation enhances sympathetically controlled ejaculatory mechanisms, while inhibiting parasympathetically mediated erectile responses (Hull et al., 1989; Hull et al., 1999). Different levels of extracellular DA then may aid in controlling the timing of copulatory events by acting through the two DAR subtypes (Hull et al., 1999). Low to moderate amounts of DA acting on DA D1R may promote erections, with a shift in the autonomic balance to favour ejaculations via an increased amount of DA stimulating DA D2R. This "spillover" effect creates a mechanism through which interplay between the two DAR subtypes in the mPOA helps control the timing of male sexual behaviour.

Hormonal Effects on Dopamine

Estrogen has been shown to affect DA and DAR in sex-relevant brain regions. For example, DA is known to play a role in the caudate putamen (CP) during female copulation (e.g., Pfaus, Damsma, Wenkstern, & Fibiger, 1995; Becker, Rudick, & Jenkins, 2001), an effect hypothesized to enhance pacing behaviour (Becker, 1999), and DA has been found to interact with E2 in doing so (e.g., Mermelstein & Becker, 1995; Meredith, Auger, & Blaustein, 1997). Estradiol has been shown to affect DA and its receptors in the rat striatum in various ways, both naturally through fluctuation over the estrous cycle (Di Paolo, Falardeau, & Morissette, 1988; Levesque, Gagnon, & Di Paolo, 1989) as well as via administration to OVX females (for review, see Hruska, 1985; Becker, 1999).

DA release in the mPOA has been examined in response to sexual behaviour, demonstrating that hormonal administration alone is enough to increase DA release. This occurs only when P is administered in conjunction to a low dose of EB (2μg). Females primed with a higher dose of EB (20μg) did not show this response (Matuszewich, Lorrain, & Hull, 2000). The

increase in DA may help facilitate the sexual motivation of the female, a response not required when circulating gonadal hormones are extremely high.

Most studies examining the effect of estrogen on DA focus on repeated estrogen administration (e.g., Levesque & Di Paolo, 1989; Levesque & Di Paolo, 1991; Levesque & Di Paolo, 1993; Lammers, D'Souza, Qin, Lee, Yajima, & Mouradian, 1999), and the ones that used acute injections typically used doses and timings that were outside of the normal sexual priming methods used (Di Paolo, Rouillard, & Bedard, 1985; Levesque & Di Paolo, 1988; and reviewed in Hruska, 1985; Becker 1999; Sanchez, Bourque, Morissette, & Di Paolo, 2010), or used adult male rats (Hruska, 1986; Hruska & Nowak, 1988). The effect of P in combination with EB administration on DA in the CP has received much less attention. When given independently, P was found to decrease D2R four hours later, but this effect was not seen when EB was given prior. However there was an increase in D2R levels 24 hours after EB+P treatment (Fernandez-Ruiz, Amor, & Ramos, 1989).

Progesterone increases have been recorded concurrently with DA increases in areas associated with female sexual behaviour, such as the mPOA (Matuszewich et al., 2000) and the striatum (Becker et al., 2001). Conversely, in the mPOA, an area well-known to be necessary for maternal behaviour, decreases in P and DA have been seen concomitantly at the time of parturition, when the onset of maternal behaviour begins (Lonstein, Dominguez, Putnam, De Vries, & Hull, 2003). Evidence also shows that P interacts with DA in other brain areas important for female sexual behaviour, increasing DA levels in the VTA (Frye, Bayon, & Vongher, 2000), and DA levels and D1R density in the striatum (Dluzen & Ramirez, 1989; Levesque & Di Paolo, 1990). Subcutaneous administration of P also leads to increased DA secretion into hypophyseal portal blood (Cramer, Parker, & Porter, 1979b), while the opposite is observed following E2 administration (Cramer, Parker, & Porter, 1979a).

Many possibilities exist for how P may act in the mPOA, as evidence demonstrates different actions in different regions important for female sexual behaviour. DAergic cells have been found to express PR in the preoptic area of female rats (Lonstein & Blaustein, 2004) and macaques (Kohama, Freesh, & Bethea, 1992) using immunocytochemical techniques, though others (i.e., Sar, 1988; Fox, Harlan, Shivers, & Pfaff, 1990) found contradictory results using autoradiographical techniques, a procedure that may underestimate PR (Sar & Stumpf, 1973). In the VMH, the binding of P to traditional intracellular progesterone receptors (PR) facilitates

lordosis (for review, see Frye, 2001). In the VTA however, the facilitation of lordosis is instead independent of PRs, through rapid actions of P at non-genomic neuronal membranes (for review, see Frye & Walf, 2008). This is complemented by findings that PRs are not observed in DAergic cells of the VTA (Sar, 1988; Lonstein & Blaustein, 2004).

Progesterone likely has a more direct interaction with DA in terms of female sexual behaviour, as it plays a role in DAR-related second messenger actions. Increased cAMP levels in the mPOA and other regions four hours following P injection have been recorded (Collado, Rodriguez-Manzo, & Cruz, 1985), and both systemic injections and direct infusions of cAMP into the brain stimulates lordosis in EB-primed rats (Beyer, Canchola, & Larsson, 1981). Meanwhile reducing cAMP degradation via administration of phosphodiesterase inhibitors also enhances lordosis in rats given low doses of P (Beyer & Canchola, 1981). Various studies by Frye and colleagues have demonstrated that the non-genomic actions of P in the VTA on lordosis also require a role for DA and its downstream components (for review, see Frye & Walf, 2008).

Steroid receptors, including PR, have been discovered to be activated by compounds other than steroid hormones through a series of in vitro studies, and also through the receipt of certain behaviourally relevant stimuli. This is referred to as ligand-independent. For example, administering vaginocervical (VCS) stimulation in the absence of P has been shown to activate PR (Auger, Moffatt, & Blaustein, 1997). Among the known extracellular compounds that are able to interact with cell membrane receptors, DA has been demonstrated to stimulate intracellular phosphorylation pathways in two separate manners (Power, Lydon, Conneely, & O'Malley, 1991a; Power, Mani, Codina, Conneely, & O'Malley, 1991b). The first was through the activation of the chicken ovalbumin upstream promoter (COUP) transcription factor (Power et al., 1991a), and also through the translocation of the PR transcription factor from cytoplasm to the nucleus when in an in vitro cell transfection system (Power et al., 1991b).

Mani and colleagues (1994) further demonstrated the ability of both a non-selective DA agonist and a selective D1R agonist to activate PR in a ligand-dependent manner, mimicking behavioural effects typically facilitated by P. This occurred following both intracerebroventricular (icv) administration and infusion to the VMH, resulting in a promotion of lordosis. This effect could be blocked by either the infusion of PR antagonists or antisense oligonucleotides to PR mRNA (Mani et al., 1994), and PR knockout mice failed to display similar effects (Mani et al., 1996), indicating the effect seen by DA administration required PR,

and providing evidence of the existence of cross-talk between signalling pathways activated by P and the DA D1R agonist SKF 38393 (Mani et al., 1994; Mani et al., 1996). Later it was discovered that an attenuation of sexual receptivity is seen in rats following infusions of anti-sense oligonucleotides that block DARPP-32, a DA- and adenosine 39,59-monophosphate (cAMP)-regulated phosphoprotein, indicating that DARPP-32 activation is necessary for sexual receptivity induced by both PR regulation and DA facilitation (Mani et al., 2000). Furthermore, although both mechanisms result in the activation of cAMP-mediated protein kinase A (PKA), which ultimately leads to the phosphorylation of DARPP-32, the interdependent signal transduction pathways initiated by both P and SKF 38393 are surprisingly independent of each other, indicating that these two mechanisms activate distinct upstream signalling kinase pathways (Mani et al., 2000). Recently it has also been demonstrated that both PR isoforms (PR-A and PR-B) are required for this DAergic ligand-dependent activation, as reduced sexual behaviour is exhibited by both PR-A and PR-B mutant mice (Mani, Reyna, Chen, Mulac-Jericevic, & Conneely, 2006). This finding has since been narrowed down to the D5R, part of the D1R-like subtype (Mani et al., 2001), and has only been found to occur in the VMH, and not the mPOA or arcuate nucleus (Apostolakis et al., 1996), despite the observation that D5R and PR are co-expressed in all three of these areas in female rats (Blaustein, Lubbers, Meredith, & Wade, 1999).

The interaction between specific ERs and DA has been much less studied. In male mice, DA activates sexual behaviour independent of ERα (Wersinger & Rissman, 2000), while in female mice, the typical facilitation of lordosis following administration of the non-specific DA agonist apomorphine (APO) is not seen in ERα-KO, but is still observed in ERβ-KO mice, however only following sex experience under EB+P primed conditions (Kudwa & Rissman, 2003). Currently, no research has examined the effects of GPER1 and DA in the rat brain (Shughrue et al., 1997).

Dopamine in the mPOA and Female Sexual Behaviour

Although DA in the mPOA has clearly been shown to control male sexual behaviour in the rat, studies examining its effect in the female have been more inconsistent, despite the fact DA release during copulation in the mPOA and striatum is identical for both male (Blackburn, Pfaus, & Phillips, 1992) and female rats (Mermelstein & Becker, 1995; Pfaus et al., 1995; Matuszewich et al., 2000). Specifically, an increase in DAergic release in the mPOA is observed

not only in response to circulating gonadal hormones, but also concurrently with copulation, although this copulatory increase is only seen in females primed with low doses of EB (Matuszewich et al., 2000). This suggests that perhaps DA is critical for the facilitating effect of P on female sexual behaviour. Sexual stimulation of any type received from the male, involving VCS (intromissions and ejaculations) or not (mounts), did not influence the increase of DA differentially (Matuszewich et al., 2000), though all three contribute perineal stimulation to the female. Although there is an increase in DA levels found in both the striatum (Mermelstein & Becker, 1995) and the nucleus accumbens (NAc) (Pfaus et al., 1995) in response to copulation, the mPOA did not show a similar increase in extracellular DA, although metabolites were observed to rise during copulation (Matuszewich et al., 2000). Finally, divergent again from the NAc (Pfaus et al., 1995), DA in the mPOA showed no change in females in response to the presence of a male alone, leading the authors to conclude that hormonal state, copulatory environment, and perineal stimulation all may contribute to altering extracellular DA levels with the mPOA of the female rat (Matuszewich et al., 2000).

Foreman and Moss (1979) were the first to directly test the role of DA in the mPOA, observing an increase in the frequency of lordosis following DAR agonist infusions; infusions of DAR antagonists decreased this behaviour. Conversely, infusions of the selective DA D1R agonist SKF 38393 to females primed only with estradiol benzoate (EB-alone) resulted in no change to lordosis (Apostolakis et al., 1996). Infusion of amphetamine, a DAR agonist, into the mPOA altered pacing levels, increasing the time it took the female to return to the male after mounts and ejaculations (Guarraci, Frohardt, Hines, Navaira, Smith, & Wampler, 2008). This is similar to the disruption in pacing and reductions in solicitations discovered following ibotenic acid lesions in the mPOA mentioned above (Hoshina, Takeo, Nakano, Sato, & Sakuma, 1994; Yang & Clemens, 2000). These paced mating changes have been hypothesized to be the result of sensitivity modifications to the sensory input provided by male sexual stimulation (Yang & Clemens, 2000), leading Guarraci and colleagues (2008) to suggest that DA in the mPOA may control female sexual behaviour through the adjustment of the female's sensitivity to copulation.

Recently we have examined the role of various DAR agonists and antagonists under partially- (EB-alone) and fully-primed (EB+P) hormonal conditions. Results indicate that the role of DA and DAR within the mPOA on female sexual behaviour is more complex than that of male sexual behaviour, going beyond the simpler dual function discovered by Hull and

colleagues (1989). Shifting the ratio of DA D1R/D2R in favour of DA D2R in EB-alone females, with the administration of the DA D2R agonist quinpirole, increased solicitations; correspondingly tilting the ratio in the other direction, through the administration of the DA D1R agonist SKF 38393, decreased them. Administration of the non-selective DAR agonist apomorphine, stimulating both receptors essentially equally, resulted in a general increase in solicitational behaviours. No effects were found on lordosis (Graham & Pfaus, 2010). This seems to signify the dominance of DA D2R activity in promoting sexual motivation in EB-alone females.

The roles of the DAR subtypes are reversed in fully-primed females. Shifting the DA D1R/D2R ratio in favour of DA D1R, by blocking DA D2R through the administration of the DA D2R antagonist raclopride, increases solicitations. Increasing the ratio in favour of DA D2R, by blocking DA D1R with the DA D1R antagonist SCH 23390, decreases them. A similar decrease is also observed following administration of the non-selective DAR antagonist flupenthixol. Similar to the agonist data, no effects were observed on lordosis (Graham & Pfaus, 2012). These results indicate that in EB+P females there is a dominance of DA D1R activity in promoting sexual motivation, while also demonstrating that the DAR ratio can be altered by the presence of P, shifting the role of DAR subtype to a predominately facilitative DA D1R activation.

Outline of the Current Thesis

The role of the current thesis was to further understand the importance of the DA D1R/D2R ratio on female sexual behaviour, and to determine if the explanations of ratio differences explaining the behavioural results are real and accurate. The DAR ratio is prominent in the male literature, reflecting differential roles in the timing of copulatory events via stimulation of the two DAR subtypes (Hull et al., 1999), and the behavioural results of our lab (i.e., Graham & Pfaus, 2010; Graham & Pfaus, 2012) imply that the ratio is important in the control of female solicitations, and thus sexual motivation, in a comparable manner. Furthermore, bremelanotide, an analogue of the naturally occurring peptide alpha-melanocyte-stimulating hormone (alpha-MSH), and which has been shown to increase solicitational behaviour, causes DA release by acting on DA D1R in the mPOA the result of which is an increase in sexual motivation in the rat (Pfaus, Giuliano, & Gelez, 2007). Recently, preliminary trials on women indicate that bremelanotide may have benefits for increasing sexual behaviour in

humans as well (Kingsberg, Clayton, & Pfaus, 2015). Since previous studies from our lab examined the administration of drugs acting directly on DAR in the mPOA (i.e., Graham & Pfaus, 2010; Graham & Pfaus, 2012), we first were interested in examining how female sexual behaviours are altered under different hormonal conditions in response to mPOA administration of ascorbic acid (AA), a compound that drives DA release, rather than via binding of postsynaptic DAR through exogenous means. Under both fully and partially primed hormonal conditions, AA caused an increase in solicitational behaviour, but had no effect on lordosis, providing further evidence that the mPOA, and the DA system within it, plays a role in the control of solicitational behaviour displayed by the female rat, and thus contributes to the timing of female sexual behaviour overall (Chapter 2).

Next, this thesis examined potential modifications to DAR in the mPOA under different hormonal manipulations, in the hopes of establishing if neural changes may reflect the previously observed behavioural differences. More specifically, assorted alterations in DAR were studied in OVX female rats under exogenous administration of steroid hormones, mimicking different hormonal priming conditions traditionally used for female sexual behaviour tests. These conditions included only vehicle (oil+oil), only estradiol (EB+oil), and estradiol plus progesterone (EB+P), with hormone injections administered 48h and 4h prior to euthanasia. These manipulations were applied to help address previously unanswered questions from the aforementioned behavioural experiments, such as what effects are occurring following EB+O versus EB+P administration, and if these effects are exclusive to DA D1R and/or DA D2R. Examination of potential pathways these effects may help comprise was also performed.

Using a variety of techniques (immunohistochemistry, Western blot, and autoradiography), it was found that when primed with EB-alone females had a lower DA D1R/D2R ratio in the mPOA, but when primed with EB+P, females have a higher DA D1R/D2R ratio in the mPOA, both in terms of indirect overall counts, protein levels, and functional number. Examination of control areas did not produce similar results (Chapter 3). These results supported previous behaviour findings, and suggest that in EB-alone conditions a DA D2R-mediated system drives female precopulatory sexual behaviour, switching to a DA D1R-mediated system when P is administered with EB. The experiments of the fourth chapter examined potential neurons these DAR changes are found on, whether those neurons themselves are altered under the same hormonal manipulations, and if these neurons (which may or may not

contain DAR) differ in projecting to brain areas associated with consummatory female sexual behaviour (Chapter 4). Besides a replication of the DAR changes seen in the third chapter, it was observed that hormonal administration modified pathways from the mPOA. This was found for both EB-alone and EB+P conditions, providing evidence of vast organizational modifications under various hormonal conditions, potentially demonstrating changes throughout the estrous cycle. In Chapter 5, the findings of the previous chapters are incorporated with previous discoveries in the literature to create a potential model of the brain circuitry that may be associated with female sexual behaviour. This model will help comprehension of the potentially multiple roles that the mPOA plays in female sexual behaviour, and how the DAR ratio within this area can help control the timing of specific behavioural displays. Specifically, this model will show the role of the DAR ratio in activating solicitations, thereby preparing the female to copulate, and how it helps control lordosis, toggling these mutually inhibited behavioural responses on and off with precision so the full display of female sexual behaviour can be exhibited. This model will help allow us to better understand how the mPOA acts as an area of integration in the many organizational pathways required for the display of such enriched behaviours, allowing for better treatment of sexual desire disorders. A general discussion is provided in the final chapter (Chapter 6).

CHAPTER 2: INFUSIONS OF ASCORBIC ACID INTO THE MEDIAL PREOPTIC AREA FACILITATE APPETITIVE SEXUAL BEHAVIOR IN THE RAT

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Abstract

Ascorbic acid (AA), also known as Vitamin C, enhances dopamine (DA) transmission in mesolimbic and nigrostriatal terminals, and augments DA-mediated behaviors. It is not yet known whether AA has a similar influence in other DA terminals, in particular terminals of the incertohypothalamic system that modulate the function of the medial preoptic area (mPOA). In female rats, DA in the mPOA plays a critical role in the generation of appetitive sexual responses, notably solicitations, hops, and darts, and we have shown previously that the role of DA in this region on female sexual behavior changes depending on the hormonal profile of the female. Since AA has often been used as a vehicle control in the examination of rat sexual behavior, the present study examined the effect of infusions of AA to the mPOA of sexual experienced ovariectomized rats under two hormonal conditions: partially-primed with estradiol benzoate (EB) alone or fully-primed with EB and progesterone. Relative to saline baselines, females under both hormonal conditions displayed a significant increase in appetitive sexual behaviors following infusions of AA. No difference in lordosis behavior was observed following AA infusions relative to saline baselines. We suggest that the mechanism by which AA infusions to the mPOA increase appetitive sexual behaviors in female rats may be through dose-dependent DA receptor interactions, possibly through both presynaptic release mechanisms and/or postsynaptic DA D1-related messenger systems.

Keywords: Ascorbic Acid, Appetitive Behaviors, Medial Preoptic Area, Female Sexual Behavior, Steroid Hormones

Introduction

L-Ascorbic acid (AA), commonly known as Vitamin C, plays a role in a number of functions within the mammalian brain, including antioxidant protection, peptide amidation, myelin formation, synaptic potentiation, and protection against glutamate toxicity (May, 2012). Although AA is typically synthesized from glucose in most animals, humans and a few other species must obtain ascorbate from their diets (Rebec & Pierce, 1994). Ascorbate uses the blood supply to reach the brain, under control of the choroid plexus. High levels of ascorbate have been found in many forebrain structures of both rats and humans, including the hypothalamus and striatum (Mefford, Oke, & Adams, 1981; Milby, Oke, & Adams, 1982).

Changes in extracellular concentrations of AA, as measured by microdialysis and voltammetry, correlate positively with motor activity (O'Neill, Grünewald, Fillenz, & Albery, 1982) and AA release increases in response to physical stimulation such as tail pinch (Boutelle, Svensson, & Fillenz, 1989), much the same way as dopamine (DA) (Girbe, Ramassamy, Piton, & Costentin, 1994; Morales, Fuentes, Ballaz, Obeso, & Rodriguez, 2012). In fact, AA in the striatum increases extracellular concentrations of DA, and by preventing DA oxidation, leads to greater extracellular diffusion (Morales et al., 2012). Conversely, inactivation of extracellular AA by infusions of ascorbate oxidase to the striatum disrupts DA-mediated behaviors, including open-field locomotion, approach toward novel objects, and social interactions with other rats (Rebec & Wang, 2001). However, whether AA potentiates or disrupts normal DA function appears to be dose-dependent. High amounts of AA can bind directly to DA receptors (DAR) (Tolbert, Morris, Spollen, & Ashe, 1992) and block DAR agonist binding dose-dependently or inhibit antagonist binding in a U-shaped curve (Heikkila, Cabbat, & Manzino, 1981; Heikkila, Cabbat, & Manzino, 1982). Behavioral evidence suggests that this postsynaptic binding causes AA to act as a DAR antagonist (Heikkila et al., 1981; Heikkila et al., 1982; Tolbert, Thomas, Middaugh, & Zemp, 1979a; Tolbert, Thomas, Middaugh, & Zemp, 1979b; Desole, Anania, Esposito, Carboni, Senini, & Miele, 1987; White, Carpenter, Block, Basse-Tomusk, Gardiner, & Rebec, 1988), particularly within the striatum (White, Maurer, Kraft, Oh, & Rebec, 1990). For example, low doses of AA potentiate amphetamine-induced conditioned place preference (Pierce, Rowlett, Rebec, & Bardo, 1995) and open field behavior (Wambebe & Sokoma, 1986), whereas higher doses have no effect. Bilateral infusions of a high dose of AA to the striatum (42 μg total) attenuated the induction of rearing, head bobbing, and sniffing, by systemic

amphetamine, and enhanced the ability of the DAR antagonist haloperidol to inhibit amphetamine-induced forepaw shuffling and locomotion (White et al., 1990). Infusions of lower doses of AA have yet to be examined, so it is not clear whether this is a dose-dependent effect on AA on postsynaptic binding. Indeed, questions have been raised whether systemic AA actually gets into the brain and whether peripheral mechanisms (e.g., stimulation of sympathetic nervous system activity) can account for the increased DA transmission (Rebec & Pierce, 1994).

AA is often used to help dissolve drugs for administration both systemically and via infusion, and is therefore also used itself as a vehicle control. For example, vehicles consisting of 0.2% AA dissolved in sterile water have been used for experiments examining the effects of different DA drugs on male sexual behavior, including the non-selective DAR agonist apomorphine (Hull, Bitran, Pehek, Warner, Band, & Holmes, 1986; Sachs, Akasofu, & McEldowney, 1994; Dominguez, Riolo, Xu, & Hull, 2001) and DAR antagonist flupenthixol (Pehek, Warner, Bazzett, Bitran, Band, Eaton, & Hull, 1988), selective DA D1R agonists SKF-82526 (Hull, Warner, Bazzett, Eaton, Thompson, & Scaletta, 1989) and dihydroxyphenyl-tetrahydrothienopyridine (THP) (Markowski, Eaton, Lumley, Moses, & Hull, 1994), selective DA D1R antagonist SCH-23390, the DA D3R/D2R agonist quinelorane, and the DA D2R antagonist raclopride (Moses, Loucks, Watson, Matuszewich, & Hull, 1995). AA control solutions have been injected subcutaneously (i.e., Sachs et al., 1994) or infused into brain regions, including the medial preoptic area (mPOA), with no apparent alteration in male sexual behavior. However, we recently observed an increase in female appetitive sexual behaviors following a single infusion of an AA vehicle to the mPOA. Because the mPOA is a critical region in the control of appetitive sexual behaviors in females, including solicitations and hops and darts (Hoshina, Takeo, Nakano, Sato, & Sakuma, 1994), and because incertohypothalamic DA release in the mPOA is a key factor in the stimulation of appetitive sexual responses in females (Pfaus, 2009; Pfaus, Giuliano, & Gelez, 2007; Graham & Pfaus, 2010; Graham & Pfaus, 2012), we examined the effects of intra-mPOA infusions of AA on both appetitive and consummatory aspects of female sexual behavior in two hormone-treatment conditions, one in which females were primed fully with estradiol benzoate (EB) and progesterone (P), and another in which females were primed partially with EB alone. It was hypothesized that the dose of AA, corresponding to what has been typically used as a solvent, would increase female rat appetitive behaviors while having no effect on consummatory behaviors.

Material and methods

Subjects

Six week old Long-Evans rats (N = 28), weighing 150-200g (females) and 200-250g (males), were obtained from Charles River Canada, Inc. (St-Constant, QC). Females were first pair-housed with wood-chip bedding in Plexiglas cages until cannulation, when they were then housed individually. Males were housed in large Plexiglas cages four to a cage, also with wood-chip bedding. All rats were housed in the same reversed 12 hour light/dark cycle colony room (lights off at 0800), given ad libitum tap water and regular rat chow, and kept in constant temperature at 21ºC.

Surgeries and sex training

Bilateral ovariectomies were performed on all females via a lumbar incision so that impregnation was impossible. This also allowed for hormone levels to be controlled throughout testing. General anaesthesia was induced with ketamine hydrochloride (100 mg/ml) and xylazine hydrochloride (20 mg/ml), mixed in a 4:3 ratio, respectively, and administered intraperitoneally (ip) at a dose of 1 ml/kg. One week of recovery was provided prior to the onset of behavioral testing.

Females were given four 30-minute training sessions of sexual experience paired with a random sexually vigorous male in a bilevel chamber (Pfaus, Smith, & Coopersmith, 1999) to equate them for sexual experience. Sessions took place at 4-day intervals to approximate the normal ovulatory cycle of the female. Females were primed with EB (10 μg) 48 hr and P (500 μg) 4 hr before each test. Steroids were administered subcutaneously in 0.1 ml of reagent-grade sesame oil (Sigma-Aldrich, St. Louis, MO).

Following sexual experience training, females were bilaterally cannulated into the mPOA. Sodium pentobarbital (60 mg/ml) was used as the general anaesthetic, administered ip in a volume of 1 ml/kg. Rats were then implanted with a 22-gauge stainless steel bilateral guide cannula aimed 1 mm above the mPOA (AP -0.6, ML ±0.5, DV -7.0 mm from bregma, incision bar set at 0) (Paxinos & Watson, 1998), with 28-gauge cannula blockers in place, cut 0.5 mm below the cannulae. Infusion cannulae, also 28-gauge, were cut 1 mm longer than the guide cannulae. All cannulae equipment was obtained from Plastics One (Roanoke, VA). A one week recovery period was then provided to the females before any infusions or testing took place.

All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Hormone and drug administration

Following the recovery period, two groups of females were tested under either EB+P (n = 15) or EB-alone (n = 13) conditions. In both conditions females were injected subcutaneously with EB (10 µg) in 0.1 ml of sesame oil 48 hr before each experimental test. Females in the EB+P group also received a subcutaneous injection of P (500 µg) in 0.1 ml of sesame oil 4 hours before every experimental test; those tested under EB-alone conditions received no injection at this time.

Females in each group were given three experimental tests with a random, sexually experienced male, with the first and last tests comprising two baselines following an infusion of physiological saline. The middle test was a drug trial where females received an infusion of 2 ng of ascorbic acid. Ascorbic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline for a final concentration of 0.2%. All infusions were done at a rate of 0.5 µl/min per side for one minute using an infusion pump (Harvard Apparatus, Pump 22), for a total volume of 1µl. Infusion cannulae were left in place for one minute following infusion to allow for absorption, after which cannulae were removed, the guide capped, and animals placed into bilevel chambers for testing.

Behavioral tests

As in the training sessions, females were placed in a bilevel chamber with a sexually vigorous male for a 30-min test immediately following the drug or vehicle infusion. The number of appetitive behaviors (solicitations, hops, darts), pacing behaviors (level changes during the test), defensive responses (rearing, kicks, boxing postures), lordosis quotients and reflex magnitudes, and male sexual behaviors (mounts, intromissions, ejaculations) were scored during each test, as previously described (Graham & Pfaus, 2010; Graham & Pfaus, 2012). Following the second saline vehicle test, females were perfused intracardially, and their brains extracted to confirm cannulae placement.

Perfusions and histology

Perfusions and placement verification were conducted as previously described (Graham & Pfaus, 2010; Graham & Pfaus, 2012). The criterion for exclusion from statistical analyses was set so that rats with both injector cannulae ending outside the boundaries of the mPOA were

exempt from the study. The end result of this was that only animals that had correct unilateral or bilateral cannulations to the mPOA were included in the analyses. Combined cannulae placement data from subjects included in the statistical analyses are shown in Figure 1.

Statistical analyses

A one-sample repeated measures analyses of variance (ANOVA) was performed on both male and female sexual behaviors. Greenhouse-Geisser corrections were made when the assumption of sphericity was not met, as per Mauchly's Test of Sphericity. All behaviors tested had three levels examined: the first saline baseline trial, the AA drug trial, and the second saline baseline trial. For each significant main effect, Bonferroni post-hoc comparisons of the individual means were made.

Results

General observations

No unusual behaviors (e.g., locomotor stimulation or inhibition) were observed in animals of either hormone condition following any infusion. Animals were easy to handle and behaved normally before and after testing. Although not analyzed statistically, differences in sexual behaviors between the EB+P and EB-alone control groups (e.g., in appetitive behaviors, defensive responses, and lordosis) were expected given previous studies of these hormone treatment regimens in bilevel chambers (Pfaus et al., 1999).

Effect of AA in females primed with EB+P or EB-alone

As seen in Figures 2 and 3, a significant main effect of AA was detected on the number of appetitive behaviors displayed during the copulation test for females primed with either EB+P ($F(1.411, 19.760) = 9.292$, $p = .003$, $\eta_p^2 = .399$) or EB-alone ($F(2, 24) = 9.528$, $p = .001$, $\eta_p^2 = .443$). In both hormonal conditions, Bonferroni post hoc analyses revealed that the dose of AA significantly increased appetitive behaviors compared to the saline baseline trials. No other changes in sexual behavior were observed.

Discussion

The present study demonstrated that AA infusions directly into the mPOA increase appetitive sexual behaviors in female rats both fully-primed with EB+P and partially-primed with EB-alone. Doses of 2 ng of AA per 1 µl increased appetitive behaviors (i.e., solicitations,

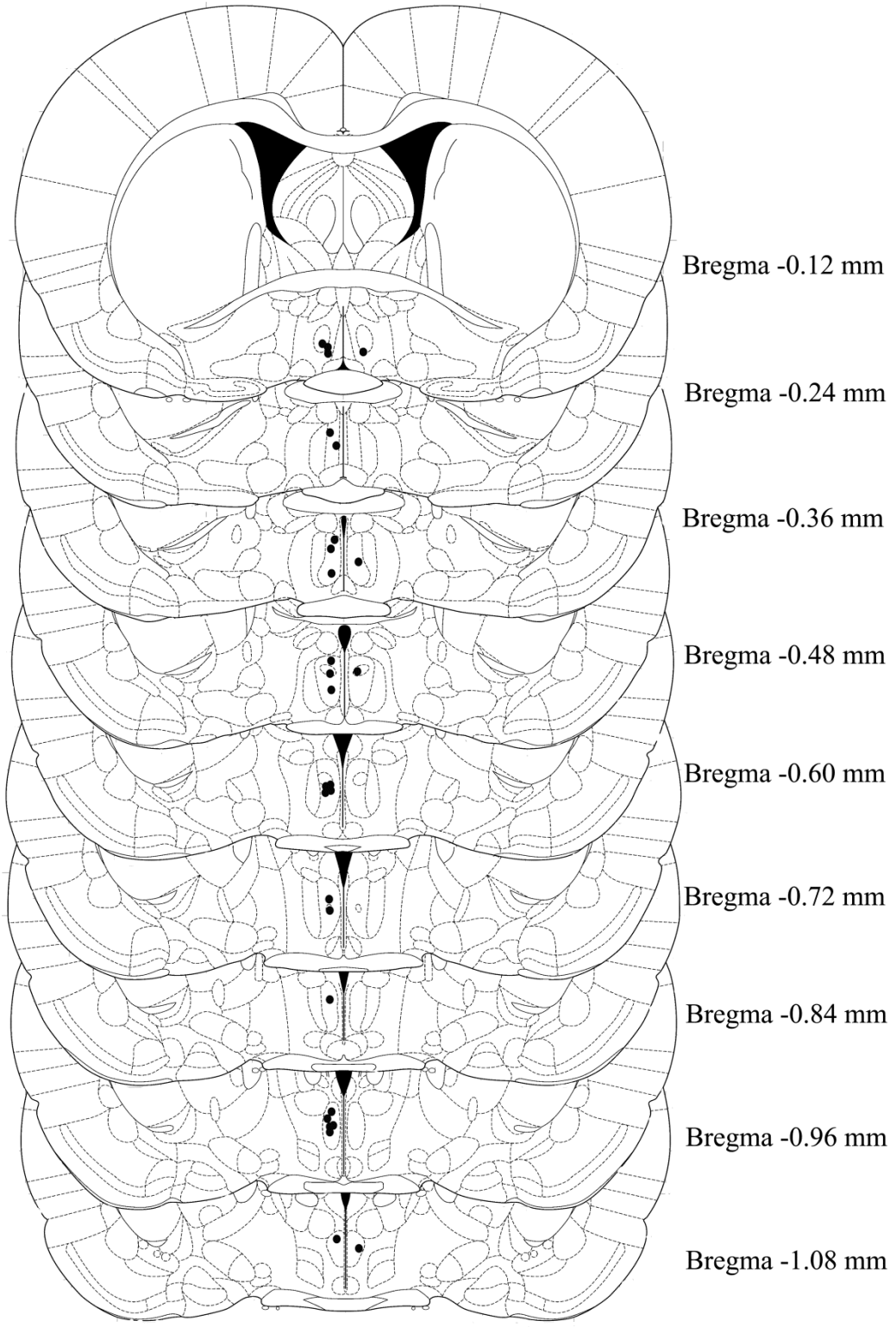
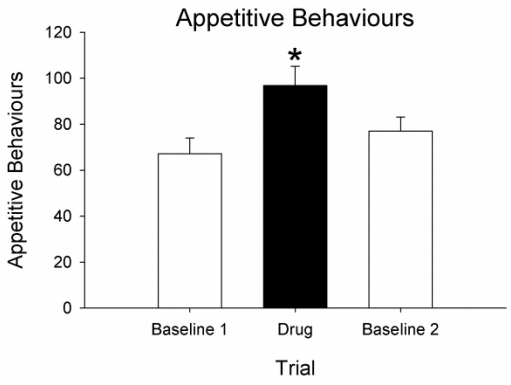
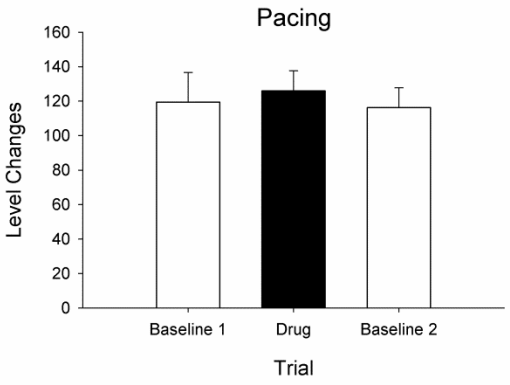
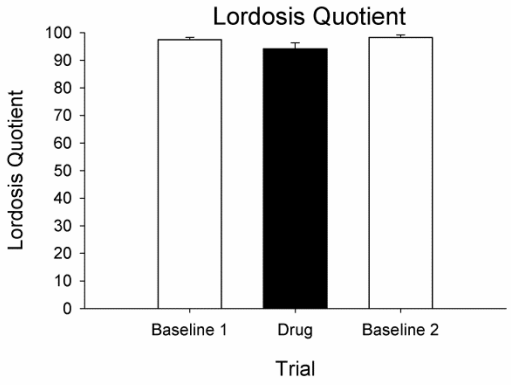
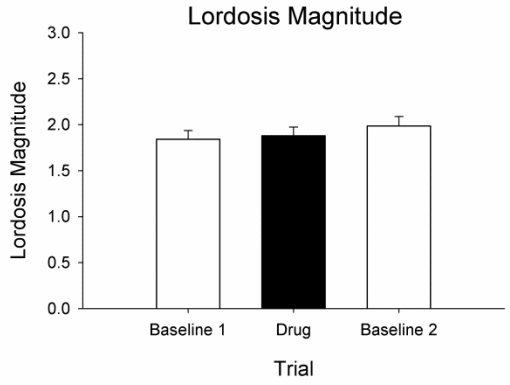


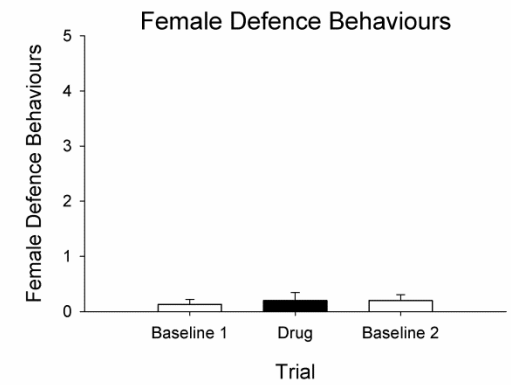
Figure 1. Cannula placements in the present experiment. Both bilateral (left side) and unilateral (right side) placements are depicted (Paxinos & Watson, 1998)**.**

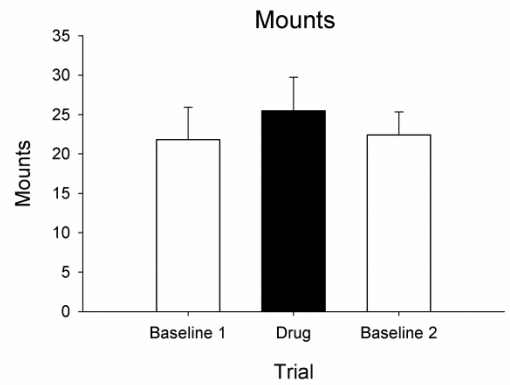

Appetitive Behaviours
Appetitive Behaviours
120
100
80
60
40
20
0
*
Baseline 1
Drug
Baseline 2
Trial

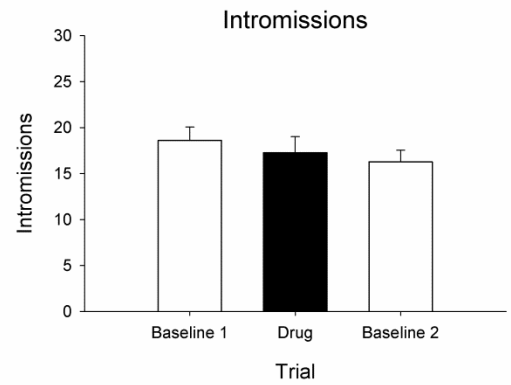

Pacing
Level Changes
160
140
120
100
80
60
40
20
0
Baseline 1
Drug
Baseline 2
Trial


Lordosis Quotient
Lordosis Quotient
100
90
80
70
60
50
40
30
20
10
0
Baseline 1
Drug
Baseline 2
Trial


Lordosis Magnitude
Lordosis Magnitude
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Baseline 1
Drug
Baseline 2
Trial


Female Defence Behaviours
Female Defence Behaviours
5
4
3
2
1
0
Baseline 1
Drug
Baseline 2
Trial


Mounts
Mounts
35
30
25
20
15
10
5
0
Baseline 1
Drug
Baseline 2
Trial


Intromissions
Intromissions
30
25
20
15
10
5
0
Baseline 1
Drug
Baseline 2
Trial

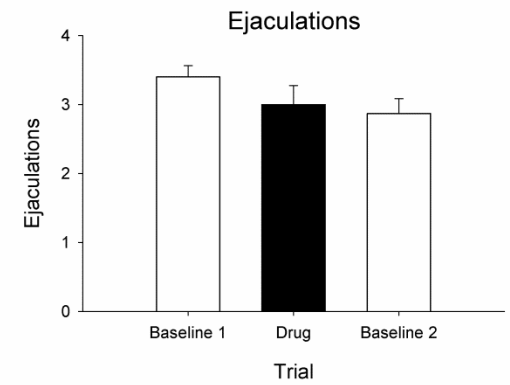
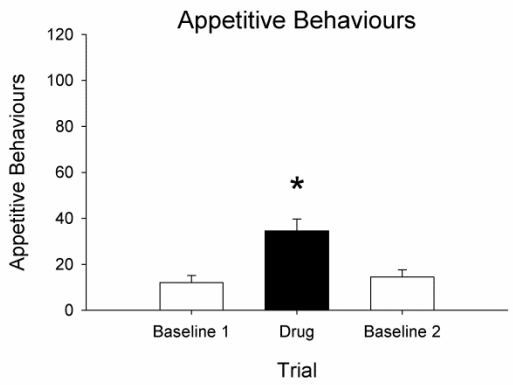
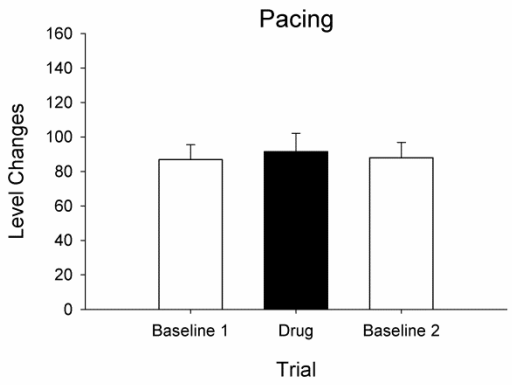
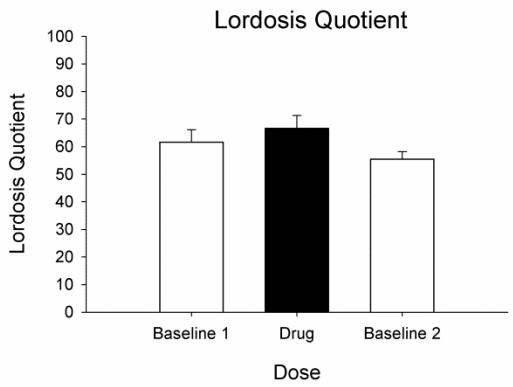
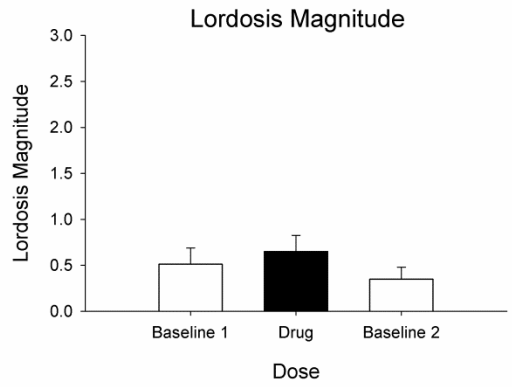

Ejaculations
Ejaculations
4
3
2
1
0
Baseline 1
Drug
Baseline 2
Trial

Figure 2. The effect of ascorbic acid or saline vehicle infusions on the mean number of appetitive behaviors, level changes, mean lordosis quotient, mean lordosis magnitude, defensive behaviors, male mounts, male intromissions, and male ejaculations, in females primed with EB+P. Error bars represent the standard errors (* = $p < .05$).


Appetitive Behaviours
Appetitive Behaviours
120
100
80
60
40
20
0
*
Baseline 1
Drug
Baseline 2
Trial


Pacing
Level Changes
160
140
120
100
80
60
40
20
0
Baseline 1
Drug
Baseline 2
Trial


Lordosis Quotient
Lordosis Quotient
100
90
80
70
60
50
40
30
20
10
0
Baseline 1
Drug
Baseline 2
Dose


Lordosis Magnitude
Lordosis Magnitude
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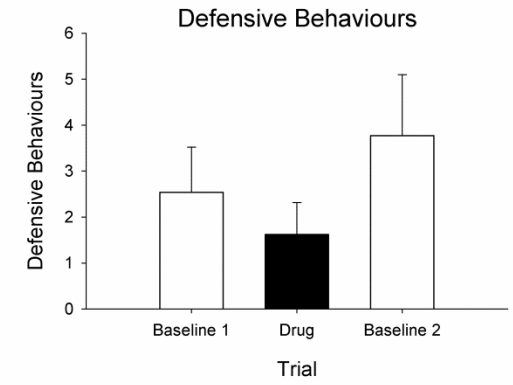
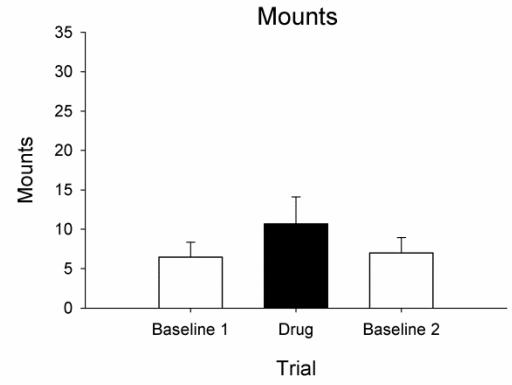
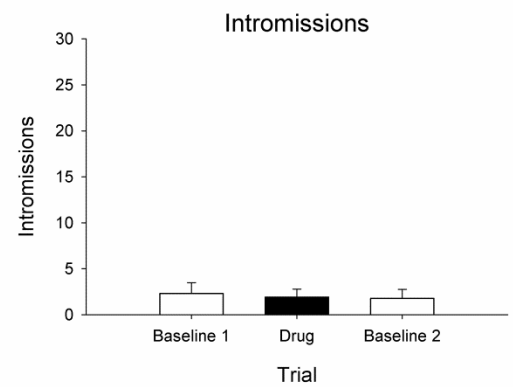
2.5
2.0
1.5
1.0
0.5
0.0
Baseline 1
Drug
Baseline 2
Dose


Defensive Behaviours
Defensive Behaviours
6
5
4
3
2
1
0
Baseline 1
Drug
Baseline 2
Trial


Mounts
Mounts
35
30
25
20
15
10
5
0
Baseline 1
Drug
Baseline 2
Trial


Intromissions
Intromissions
30
25
20
15
10
5
0
Baseline 1
Drug
Baseline 2
Trial

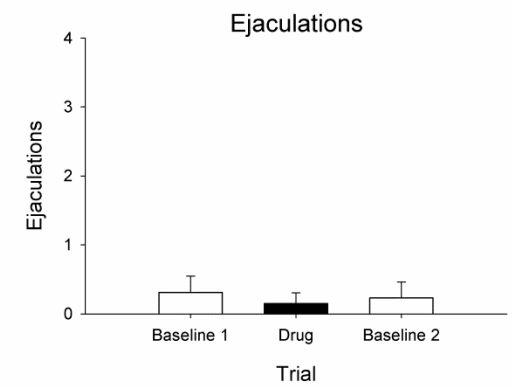

Ejaculations
Ejaculations
4
3
2
1
0
Baseline 1
Drug
Baseline 2
Trial

Figure 3. The effect of ascorbic acid or saline vehicle infusions on the mean number of appetitive behaviors, level changes, mean lordosis quotient, mean lordosis magnitude, defensive behaviors, male mounts, male intromissions, and male ejaculations, in females primed with EB-alone. Error bars represent the standard errors (* = $p < .05$).

hops, darts) compared to the two saline baseline trials. No other measures of female sexual behavior, including lordosis, were affected by AA administration. This is particularly noteworthy as past studies have used this concentration of AA as a solvent to infuse drugs for analyzing rat sexual behavior. Given the current findings, it is possible that this concentration of AA could have had effects of its own.

As noted in the introduction, a number of DAR agonists, notably the nonselective DAR agonist apomorphine, have been infused into the brain using a 0.2% AA concentration (e.g., Hull et al., 1986; Hull et al., 1989; Moses et al., 1995). Most of the research examining the neural interactions of AA and DA has focused on the nucleus accumbens (Pierce & Rebec, 1992). Studies have used both voltammetry and in vivo microdialysis to examine AA release in response to pharmacological challenges. For example, Liu and colleagues (2000) found that AA levels in the nucleus accumbens of both intact and 6-OHDA-lesioned rats were increased by the DA D1R antagonist SCH 23390, but decreased following administration of the DA D2R antagonist sulpiride. This pattern of activity was suggested by the authors to indicate that AA release in the ventral striatum comes about as a result of either DA D1R blockade or DA D2R activation, and any changes that are seen due to administration of indirect DAR agonists are mainly the result of DA D2R activation (Liu, Wu, Huang, & Xiao, 2000). This disparity in effects between DA D1R and DA D2R is similar to the manner in which those receptors in the mPOA control appetitive sexual behavior in ovariectomized rats primed with EB-alone (Graham & Pfaus, 2010). However, in the presence of EB+P we have shown that DA D1R are critical for appetitive sexual behavior in female rats (Graham & Pfaus, 2012). The effects of AA may possess dose-dependent actions in terms of DAR interaction (Wambebe & Sokomba, 1986), and it is possible that DAR in the mPOA may interact with the actions of AA differently from the ventral striatum.

A lack of consistent findings relating to AA synaptic modulation does not preclude that AA is related to the stimulation of DA release directly, or via other mechanisms including, but not limited to, glutamate-mediated neurotransmission (Phebus, Roush, & Clemens, 1990). Indeed the extracellular level of AA in the striatum exceeds that of DA by as much as 1000 times (Justice, 1987; Adams, 1990). Actions of AA on a number of second-messenger substrates related to DAR actions (e.g., inhibition of adenylate cyclase activity, as occurs with agonist activity at populations of DA D2R linked to Gi proteins) (Thomas & Zemp, 1977; Tolbert et al.,

1979a) may also play a role in its enhancement of DA action. Thus, AA may alter synaptic functions of DA by affecting both presynaptic release mechanisms and postsynaptic second messenger systems (Rebec & Pierce, 1994).

Glutamate and DA both have been shown to affect the release of AA, and in turn both appear to be regulated to some degree by this release (see Rebec & Pierce, 1994). It is therefore possible that interactions between glutamate and AA could account for the inhibitory effects on DA found by high doses of AA. Unfortunately, not much is known as to the role of glutamate, if any, after administration of low doses of AA, as used in the current study. However, glutamate in the mPOA is an important neurotransmitter in the control of male sexual behavior. Administration of glutamate by microinjection or reverse-dialysis facilitates male copulation (Giuliano, Rampin, Brown, Courtois, Benoit, & Jardin, 1996; Dominguez, Gil, & Hull, 2006; and reviewed in Hull & Rodriguez-Manzo, 2009), and blocking N-methyl-D-aspartate (NMDA) receptors in the mPOA, upon which glutamate acts on, inhibits this behavior (Vigdorchik, Parrish, Lagoda, McHenry, & Hull, 2012). Additionally, in males, an increased release of DA in the mPOA by glutamate via a nitric oxide-dependent process has been shown (Dominguez, Muschamp, Schmich, & Hull, 2004).

Unfortunately little is known about glutamate's effects in the mPOA on female sexual behavior. Infusion of NMDA, an amino acid agonist that mimics the effects of glutamate, into the preoptic area of fully-primed female rats had no effect on lordosis, but infusion of AP-5, a selective NMDA antagonist, decreased that behavior (McCarthy, Curran, & Feder, 1991). Effects on appetitive behaviors are yet to be determined. Elsewhere in the brain, in areas important for female sexual behavior, infusions of glutamate to the VMH inhibits lordosis and appetitive behaviors in both EB-alone and EB+P females (Kow, Harlan, Shivers, & Pfaff, 1985; McCarthy et al., 1991; Georgescu & Pfaus, 2006a) and glutamate antagonists increased female sexual behaviors, particularly in females primed with EB-alone (Georgescu & Pfaus, 2006b). Conversely, infusions of MK-801, an NMDA receptor antagonist, to the VMH decreased lordosis quotients (Petralia, DeBold, & Frye, 2007). The opposite effect was found following infusions of MK-801 to the ventral tegmental area (VTA), another area important in female sexual behavior (Petralia et al., 2007). It may be then that the effect of AA in the mPOA on female sexual behavior may be due to altering DA function, glutamatergic function, or some sort of interaction between both. This relationship should be further examined.

Other potential mechanisms may help explain how AA could affect female sexual behavior. For example, AA potentiates the inhibitory effect of DA on prolactin (PRL) release (Shin & Stirling, 1988; Shin, Stirling, Hanna, Lim, & Wilson, 1990), and PRL has been shown to affect female sexual behaviour, though contradictory results have been demonstrated with PRL having both facilitatory (Harlan, Shivers, & Pfaff, 1983; Drago & Lissandrello, 2000) and inhibitory (Dudley, Jamison, & Moss, 1982) effects. Additionally, AA modulates noradrenergic systems (Paterson & Hertz, 1989; Kimelberg & Goderie, 1993), and norepinephrine plays an excitatory role in sexual desire (reviewed in Pfaus, 2009). Thus, AA may potentiate the effects of DA at hypothalamic, limbic, and cortical levels to alter mood, emotion, and sexual function.

Although no effects of AA were found when it was used as a vehicle in studies of male sexual behavior, the current study found a clear role for AA in increasing female sexual behavior. This raises the question whether a gender-specific effect of AA exists. Interestingly, a human study has shown that AA treatment (3000 mg/day) increases mood in both genders, but only increases rates of penile-vaginal intercourse in women, an effect that was not found for men (Brody, 2002). This effect was specific to penile-vaginal intercourse, and not on other types of sexual behavior such as masturbation, leading the author to suggest that the mechanism at work is a central one, motivating women to actively seek out intercourse (Brody, 2002). This may be analogous to the findings of the current study, where AA administration increased appetitive behaviors in the female rat, while having no effect on consummatory behaviors. This effect is similar to that following administration of DA or its receptor agonists into the mPOA, and thus may be a result of augmented DA transmission in the mPOA, or via some other sort of interaction between DA and AA, that preferentially stimulates appetitive sexual behaviors in females. Combined with the study by Brody (2002), the results of the current study support the idea that DA in the mPOA of females is driving desire for copulation, whereas in male rats (and men) it does less to drive this motivation, or requires more DA release to do so, as previous studies focusing on male sexual behavior have not found a change in behavior when administering AA alone as a vehicle solution (Pehek et al., 1988; Hull et al., 1989; Markowski et al., 1994; Moses et al., 1995; Dominguez et al., 2001).

CHAPTER 3: OVARIAN STEROIDS ALTER DOPAMINE RECEPTOR POPULATIONS IN THE MEDIAL PREOPTIC AREA OF FEMALE RATS: IMPLICATIONS FOR SEXUAL MOTIVATION, DESIRE, AND BEHAVIOUR

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Graham, M.D., Gardner Gregory, J., Hussain, D., Brake, W.G., & Pfaus, J.G. (2015). Ovarian steroids alter dopamine receptor populations in the medial preoptic area of female rats: Implications for sexual motivation, desire, and behaviour. ***European Journal of Neuroscience, 42*****(12), 3138-3148. doi: 10.1111/ejn.13121**

Abstract

Dopamine (DA) transmission in the medial preoptic area (mPOA) plays a critical role in the control of appetitive sexual behaviour in the female rat. We have shown previously that a D1R-mediated excitatory state appears to occur in females primed with estradiol benzoate (EB) and progesterone (P), whereas a D2R-mediated inhibitory state appears to occur in females primed only with EB. The present experiment employed three techniques to better understand what changes occur to DA receptors (DAR) in the mPOA under different hormonal profiles. Ovariectomized females were randomly assigned to one of three steroid treatment groups: EB+P (10μg and 500μg, respectively), EB+Oil, or the control (Oil+Oil), with hormone injections administered 48h and 4h prior to euthanasia. First, the number of neurons in the mPOA that contained D1R or D2R was assessed using immunohistochemistry. Then the mPOA and two control areas (the prelimbic cortex and the caudate putamen) were analysed for DAR protein levels using Western blot, and DAR functional binding levels using autoradiography. Ovarian steroid hormones affected the two DAR subtypes in opposite ways in the mPOA: all three techniques support previous behavioural findings that females primed with EB-alone have a lower D1R/D2R ratio, and thus a D2R-mediated system, and females primed with EB+P have a higher D1R/D2R ratio, and thus a D1R-mediated system. This provides strong evidence for a dopamine-driven pathway of female sexual motivation, desire, and behaviour that is modified by different hormone priming regimens.

Keywords: Dopamine Receptors; Medial Preoptic Area; Ovarian Hormones; Western Blot; Autoradiography;

Introduction

Dopamine (DA) in the medial preoptic area (mPOA) plays a critical role in the sexual motivation and behaviour of male and female rats (Hull & Dominguez, 2007; Graham & Pfaus, 2010; Graham & Pfaus, 2012). In males, pharmacological stimulation of D1 receptors (D1R) facilitates erection and intromission, whereas stimulation of D2 receptors (D2R) causes a change from parasympathetic to sympathetic outflow, resulting in the activation of the ejaculatory mechanism (Hull et al., 1989; Hull et al., 1992; reviewed in Hull et al., 1995). Hull and colleagues proposed a DA receptor (DAR) ratio effect that controls the timing of copulation in male rats, with D1R activation regulating the initiation and timing of erections (and thus intromissions) and D2R activation controlling ejaculation onset (Hull et al., 1989; Hull et al., 1995; Hull & Dominguez, 2007).

We have recently found a similar, although more complex, relationship of DAR activation in the mPOA critical for the expression of solicitations in female rats (Graham & Pfaus, 2010; Graham & Pfaus, 2012). The results suggest a shift in DAR activation in the mPOA with hormone priming that alters their control of appetitive responses. In females primed with EB+Oil D2R activation facilitates and D1R activation inhibits sexual motivation and behaviour, whereas in females primed with EB+P, the opposite occurs. Thus, it appears that the D1R/D2R ratio in the mPOA, and its behavioural effects, is altered by the sequential addition of P to estradiol priming, which shifts the balance of DAR activation to a population of D1R that are excitatory for sexual behaviour.

To elucidate the mechanism behind the switch in D1R/D2R ratio activation three methods were utilized examining three hormonal conditions (EB+P, EB+Oil, Oil+Oil). In the first experiment, immunohistochemistry was used to compare DAR expression in the mPOA of ovariectomized rats. Western blots were used in the second experiment to examine protein levels of the DAR subtypes in three brain areas: the mPOA, the prelimbic cortex (PLC), and the caudate putamen (CP). These latter two regions were chosen as control areas due to their abundance of DAR levels (Vincent, Khan, & Benes, 1993; David, Ansseau, & Abraini, 2005). In the third experiment, functional binding levels of DAR subtypes were analyzed in the same brain areas under the same hormonal manipulations using quantitative receptor autoradiography. We hypothesized that the mPOA of females treated with EB+P would show a greater amount of D1R, as evidenced by more stained neurons, an increase in D1R protein levels, and an increase

in relative binding densities, while female rats treated with EB+Oil would show a greater amount of D2R in the mPOA. The CP was examined as a potential positive control given reports of alterations in DAR in the CP following chronic administration of estradiol (e.g., Hruska, Ludmer, & Silbergeld, 1980; Hruska, 1986; Hruska & Nowak, 1988; Levesque & Di Paolo, 1989) and progesterone (Fernandez-Ruiz et al., 1989), while the PLC was chosen as a potential negative control because alterations of DAR in this region due to hormonal administration has yet to be reported.

Materials and Method

Animals and surgery

Adult female Long-Evans rats, weighing approximately 350g were obtained from Charles River Canada, Inc. (St.-Constant, QC). Rats were pair-housed in Plexiglas cages with wood-chip bedding, with ad libitum access to regular rat chow and water. All rats were housed in the same colony room with the temperature kept constant at 21ºC, on a reversed 12 hour light/dark cycle, with lights off at 0800.

Bilateral ovariectomies were performed on females via a lumbar incision so that hormone levels could be controlled. Anaesthesia consisting of ketamine hydrochloride (100mg/ml) and xylazine hydrochloride (20mg/ml) mixed in a 4:3 ratio, respectively, was administered intraperitoneally to females at a dose of 1ml/kg. Rats were given one week of recovery following this procedure, where they were then used as stimulus females in unrelated behavioural studies within the lab, becoming sexually experienced and undergoing approximately equal amounts of sexual bouts.

Following their completion of the unrelated sexual behaviour studies, all rats were given at least a three week washout period so any prior hormonal administration would subside. Females were then randomly assigned to one of three hormonal groups: EB+P, EB+Oil (EB+O), or Oil+Oil (O+O). Estradiol benzoate (1,3,5(10)-Estratriene-3,17b-diol 3-benzoate, Cat#50-50-0) and progesterone (4-Pregnene-3,20-dione, Cat#57-83-0) were obtained from Steraloids (Newport, RI). Each rat received two subcutaneous hormonal injections before euthanasia and brain extraction; first was 0.1ml of sesame oil with or without EB (10µg) 48 hours prior, and the second was 0.1ml of sesame oil with or without P (500µg) 4 hours prior to euthanasia.

All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Immunohistochemistry procedure

Rats (n = 5 for each group) were euthanized 4 hours after their second injection (oil with or without P) via an overdose of sodium pentobarbital (120mg/kg, i.p.), and then underwent an intracardial perfusion with approximately 300ml of ice-cold phosphate-buffered saline and 4% paraformaldehyde in 0.1M of phosphate buffer. Brains were removed, postfixed for 4 hours in 4% paraformaldehyde in 0.1M of phosphate buffer and stored for 48 hours in a 30% sucrose solution at 4ºC.

Coronal slices of the mPOA (corresponding to plates 34-44 in Paxinos & Watson, 2005) were taken at 30µm sections. Sections were incubated with hydrogen peroxide for 30 minutes at room temperature, preblocked in normal goat serum (NGS) with triton TBS for two hours at room temperature, and incubated for 72 hours at 4ºC with the primary antibody recognizing either D1R (1:1000; Millipore; Cat#AB9421) or D2R (1:1000; Millipore; Cat#AB5084P) with NGS in 0.05% triton TBS. After the primary incubation phase, sections were incubated for one hour at 4ºC with the secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate (1:10000; Bio-Rad; Cat#170-6515) with NGS in 0.2% triton TBS, and incubated for two hours at 4ºC with avidin-biotinylated-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, diluted 1:55) in 0.2% triton TBS. Sections were rinsed in TBS for three rounds of five minutes each in between each incubation session. Finally, the sections were washed for 10 minutes in 50 mM Tris buffer (pH 7.6), incubated for 10 minutes at room temperature in 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris buffer (pH 7.6), then with DAB/3% hydrogen peroxide in 50 mM Tris buffer (pH 7.8) with 8% nickel chloride for 10 minutes at room temperature, where the DAB reaction was stopped by transferring sections to cold TBS at room temperature. Sections were mounted onto gel-coated slides, allowed to dry, and dehydrated in alcohol (70%, 90%, 100%, 10 minutes each) followed by immersion in Xylene for two hours. Slides were coverslipped, allowed to dry overnight, and examined using a Zeiss light microscope.

Cells that showed staining for either D1R or D2R were counted unilaterally across the mPOA for rats in each group at 80X magnification. The mPOA was defined using the pre-established landmarks in the Paxinos and Watson (2005) atlas permitting inclusion of the entirety of areas relevant to the mPOA and its subdivisions. Blind counts were performed using ImageJ software (http://imagej.nih.gov/ij/) and the Find Maxima function with a Noise Tolerance value of 35. Standardized counts were calculated for individual IHC runs, to ensure that multiple IHC

runs could be combined. These standardized counts were then averaged across each section taken of the mPOA for each animal.

Western blot procedure

Four hours following the second hormone injections (oil with or without P) animals (*n* = 8-10 per group) were stunned on dry ice and decapitated. Brains were quickly removed, flash frozen in isopentane and stored at -80ºC until tissue extraction.

Tissue extraction was done via 200µm coronal slices on a cryostat, where tissue punches (1mm in diameter) were taken bilaterally throughout the mPOA, CP, and PLC (12 punches in total in each area), and stored in Eppendorf tubes at -80ºC.

The protein in each sample was quantified through the addition of 75µl of lysis buffer, followed by three rounds of sonication lasting five seconds each, with samples kept on ice between rounds. After sonication each sample alternated flash freezing and heating, using liquid nitrogen and a heat block set at 37ºC, respectively. Samples were then centrifuged for 30 minutes at 4ºC following three rounds of this. The pellets formed by centrifugation were removed from the sample, and the protein content of the supernatant was quantified through comparison to a bicinchoninic acid assay kit (Thermo Scientific - Pierce Protein Research Products). Supernatant was then stored at 70ºC until ready for Western blot analysis.

Samples consisting of 20µg of protein, in a final volume of 25µl, were incorporated into a homemade 10% Tris-HCl gel via electrophoresis at 4V over approximately 90 minutes. Included in each gel was 2µl of Precision Plus Protein Western C Standard (Bio-Rad; Cat#161-0376) for band weight comparison. After electrophoresis proteins were transferred onto a nitrocellulose membrane (Invitrogen) using constant current at 25V for 90 minutes, on ice to prevent overheating. Upon completion the transfer membranes were separated between 50 and 37kDa using a razor blade so that primary antibodies and loading controls could be run separately. Both membrane pieces were then blocked with 5% milk in a TBS-Tween-20 (TBST) solution for one hour at room temperature and incubated overnight at 4ºC with their respective primary antibodies. The top piece of the membrane, containing the 50kDa band and higher, was incubated with antibodies recognizing protein for either D1R (1:1000; Millipore; Cat#AB1765P) or D2R (1:1000; Millipore; Cat#AB5084P) in a 5% milk-TBST solution. The bottom piece, containing the 37kDa band and lower, was used as a loading control and incubated with an

antibody recognizing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein (1:2500; Abcam; Cat#AB9485) in a 5% milk-TBST solution.

Following the overnight incubation both membrane pieces were washed seven times in TBST for five minutes each, and incubated in darkness for one hour at room temperature with the secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate (1:10000; Bio-Rad; Cat#170-6515) and a ladder secondary (Precision Protein StrepTactin-HRP Conjugate; 1:6000; Bio-Rad; Cat#161-0381) in a 5% milk-TBST solution. Membrane pieces were then washed with TBST and covered with a chemiluminescence detecting substrate (Cedarlane Labs, Burlington, ON) for one minute, before being developed using a Kodak Image Station 440 CF. Images were taken at increments of 10, 30, 90, 300, and 600 seconds, and the clearest image was used for analysis.

Protein levels, represented by densities, were simultaneously quantified for bands corresponding to GAPDH and either DAR subtype using the Gel Analysis method within the ImageJ software. Protein levels were standardized by dividing the densities of the DAR band by the density of the GAPDH band for each sample. Relative densities were then calculated by standardizing each experimental group (EB+O and EB+P) to the mean of the O+O group for each membrane set. This allowed for combining results across multiple membranes.

D1R and GAPDH protein levels were each represented by a single band at approximately 50 and 37kDa, respectively. D2R protein levels were represented by three bands at approximately 50, 75, and 100kDa, depicting the monomer, glycosylated monomer, and dimer of the receptor, respectively (Conrad, Ford, Marinelli, & Wolf, 2010).

Autoradiography procedure

Brain extraction was done in a manner consistent to that of the Western blots, with identical timing of hormone manipulation (n = 6 per group). Three series of 20 μm coronal slices, totalling 9 consecutive slices for each series, of the PLC (corresponding to plates 9-13 in Paxinos & Watson, 2005) and the anterior half of the mPOA/CP (corresponding to plates 34-39 in Paxinos & Watson, 2005) were taken and immediately mounted on Superfrost Plus slides (Fisher). The result of this was that series 1 consisted of slices 1-9, series 2 consisted of slices 10-18, and series 3 consisted of slices 19-27. Slides were desiccated under vacuum seal, left overnight at 4ºC, and then stored at -80ºC until autoradiography.

To start the autoradiography process, slides were removed from the freezer and defrosted at room temperature for approximately one hour. During this time, slides were outlined using a grease pencil. For D1R binding, slices were incubated in 800µl of buffer (50mM Tris HCl, 120mM NaCl, 5mM $CaCl_2$ $2H_2O$, 1mM $MgCl_2$ $6H_2O$, 5mM KCl, 30nM ketanserine, pH 7.4, 25ºC) containing 2nM [^{3}H]SCH 23390 (specific activity 81.9 Ci/mmol; PerkinElmer, Waltham, MA) for 90 minutes. Slides were then rinsed in cold buffer (4ºC) four times for five minutes each, followed by a quick rinse in dH_2O, and dried overnight at room temperature.

For D2R binding, slices were incubated in 600µl of buffer (50mM Tris HCl, 120mM NaCl, 2mM $CaCl_2$ $2H_2O$, 2mM $MgCl_2$ $6H_2O$, 5mM KCl, pH 7.4, 25ºC) containing 10nM [^{3}H]Sulpiride (specific activity 83.4Ci/mmol; PerkinElmer, Waltham, MA) for 120 minutes. Slides were then rinsed in cold buffer (4ºC) four times for two minutes each, followed by a quick rinse in dH_2O, and dried overnight at room temperature.

The next day the dried slides were set in cassettes with Amersham Hyperfilm MP (GE Healthcare), along with a calibrated [^{3}H] standard slide (American Radiolabeled Chemicals, Inc.). Exposure times for D1R binding were 6 and 12 weeks for the PLC and mPOA/CP, respectively and for D2R binding were 18 and 36 weeks for the PLC and mPOA/CP, respectively. After exposure was complete, films were developed by placing in Kodak GX Developer (Sigma-Aldrich, St. Louis, MO) for 5 minutes, following by a 30 second rinse in room temperature water, then fixed by placing in Kodak GX Fixer (Sigma-Aldrich, St. Louis, MO) for 7 minutes. Films were then rinsed under running room temperature water for one hour, and dried overnight.

Quantitative densitometry with a computerized image processing system (MCID Core Digital Imaging Software, Cambridge, UK) was used to conduct density analysis. Calibrated [^{3}H] standards were referenced so that optical density values could be converted to fmol/mg (of wet-weight tissue), and specific D1R and D2R binding was calculated for each animal (n = 6 sections). Relative binding densities were then calculated by standardizing each experimental group (EB+O and EB+P) to the mean of the control group (O+O) for each x-ray film. This allowed for the data from multiple films to be combined.

Statistical Analyses

Group averages for the IHC data were compared using a one-way ANOVA comparing the three hormonal groups (O+O, EB+O, EB+P). Any significant omnibus F-tests were then

further analyzed using a Bonferroni post hoc analysis to determine which of the hormonal groups differed. In addition, linear and quadratic trends were analyzed for both D1R and D2R staining. Analyses of D1R/D2R ratio effects were performed using log transformations, so that the data would adhere to the assumption of linearity. Effect sizes were calculated using eta squared, with a 90% confidence interval (Steiger, 2004). Due to the paucity of previous effect size reports, all effect size interpretations were compared only within the confines of the present studies (for detailed explanation see Thompson, 2007). Thus, we used as criteria .100, .200, and .300 for small, medium, and large effect sizes, respectively.

For the Western blot data, one-way ANOVAs compared the three hormonal groups (O+O, EB+O, EB+P) on the adjusted relative densities corresponding to each protein band (one band for D1R, three bands for D2R), as well as the log-transformed D1R/D2R ratio for each of the three D2R bands. Any significant omnibus F-tests were then further analyzed using a Bonferroni post hoc analysis to determine which of the hormonal groups differed. Effect sizes were calculated using eta squared, with a 90% confidence interval.

For the autoradiography data, a 2 X 3 X 3 mixed-design ANOVA was performed on each of the standardized binding density values for each of the three brain regions of interest. Within-subject factors consisted of DA receptor type (D1R, D2R) and brain series (the sequence of first 9 slices, second 9 slices, third 9 slices, averaged across sequence). The between-subject factor was that of hormonal group (O+O, EB+O, EB+P). Similarly, a 3 X 3 mixed-design ANOVA was used to compare log-transformed D1R/D2R ratios in each of the three brain areas, with brain series (the sequence of first 9 slices, second 9 slices, third 9 slices) as the within-subjects variable, and hormonal group (O+O, EB+O, EB+P) as the between-subjects variable. Greenhouse-Geisser corrections were made when the assumption of sphericity was not met, as per Mauchly's Test of Sphericity. For all ANOVAs, any significant omnibus F-test was further analyzed using a Bonferroni post hoc analysis to determine simple effects. Due to the use of mixed-design ANOVAs, effect sizes were calculated using partial eta squared, with 90% confidence intervals.

Results

Immunohistochemistry

Representative images of the DAR staining for three hormonal groups can be seen in Figure 1. Although ependymal cells expressed DA receptor staining, consistent with previous

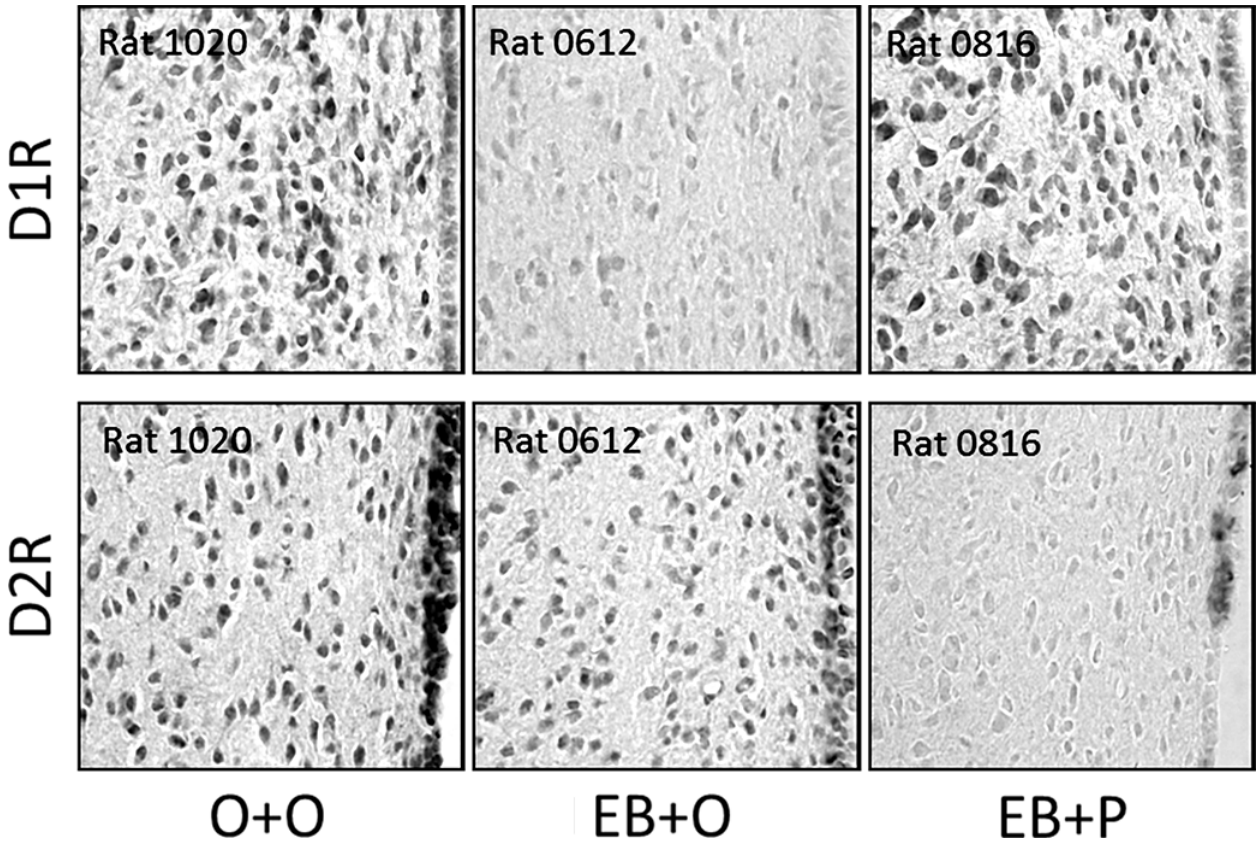

Figure 1. Immunohistochemical images of cells stained for the DAR subtypes in each of the three hormonal priming groups.

research (Tomé, Moreira, Pérez-Fígares, & Jiménez, 2007), these cells were not counted and were only used as landmarks to demarcate the mPOA.

There was a statistically significant and large overall effect of hormonal group on the number of immunoreactive cells stained for D1R ($F(2, 12) = 5.049$, $p = .026$, $\eta^2 = .457$, 90% CI [.041, .621]). Post hoc analyses revealed a significant decrease in the EB+O group compared to that of O+O, with a trend towards a decrease compared to the EB+P group. There was also a statistically significant and large quadratic trend ($F(1, 12) = 9.945$, $p = .008$, $\eta^2 = .450$, 90% CI [.086, .646]), indicating a return to control levels with the addition of P (Figure 2*A*).

No significant effect of hormonal group was found on immunoreactive cells stained for D2R ($F(2, 12) = 2.532$, $p = .121$, $\eta^2 = .297$, 90% CI [.000, .497]), though there was a medium to large effect size. However there was a statistically significant linear trend ($F(1, 12) = 5.027$, $p = .045$, $\eta^2 = .295$, 90% CI [.004, .534]), with a similarly medium to large size of effect, indicating a continuing decrease in relation to amount of hormonal priming received (Figure 2*B*).

A statistically significant effect of hormonal group on the log-transformed D1R/D2R ratio was found ($F(2, 12) = 4.065$, $p = .045$, $\eta^2 = .404$, 90% CI [.006, .582]), with a large effect size. Post hoc analyses revealed that the EB+O group had a significant decrease in log-transformed D1R/D2R ratio compared to the EB+P group, indicating that the EB+O group had a DAR ratio tilted towards D2R, while the EB+P group showed the opposite pattern (Figure 2*C*).

Western blots

A sample Western blot for each DAR subtype with corresponding protein bands and GAPDH control band can be seen in Figure 3. There was a statistically significant and large overall effect of hormonal group on D1R protein levels in the mPOA ($F(2, 25) = 6.366$, $p = .006$, $\eta^2 = .337$, 90% CI [.071, .498]). Post hoc analyses revealed a significant decrease in protein levels in ovariectomized rats treated with EB+O relative to treatment with EB+P. Hormonal manipulation also had a significant effect on all three bands relating to D2R protein levels. There was a statistically significant and very large overall effect of hormonal group on the 100kDa band ($F(2, 22) = 14.501$, $p < .001$, $\eta^2 = .569$, 90% CI [.276, .685]). Post hoc analyses revealed that EB+O protein levels were significantly increased compared to both the O+O and EB+P groups. There was also a statistically significant and medium to large overall effect of hormonal group on the 75kDa band ($F(2, 22) = 4.718$, $p = .020$, $\eta^2 = .300$, 90% CI [.031, .472]). Post hoc analyses revealed that EB+O protein levels were significantly increased compared to EB+P

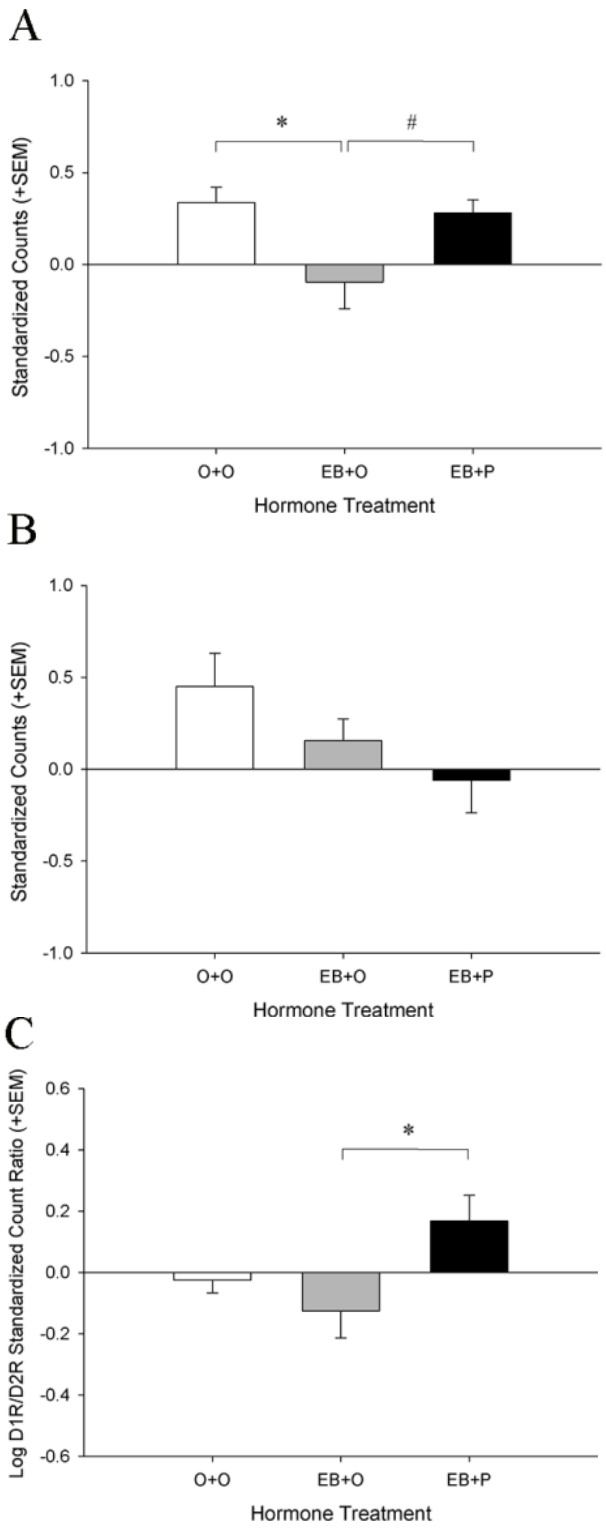

Figure 2. Immunohistochemical analyses of hormonal group on the number of immunoreactive cells stained for the DAR subtypes. Each value represents the mean standardized count ratio respective to the control (O+O) group. Error bars represent the SEM. (A) D1R staining. Females

primed with EB+O show a decreased number of immunoreactive cells stained for D1R than females primed with O+O, and a trend towards a decrease compared with females primed with EB+P. A statistically significant and large quadratic trend was also found. (B) D2R staining. There were no statistically significant differences found for the three hormonal groups, but there was a medium to large effect size, and a statistically significant linear trend was discovered. (C) Log-transformed D1R/D2R standardized count ratio. Females primed with EB+O had a significant decrease in log-transformed D1R/D2R ratio compared with females primed with EB+P. * $p < .05$; # $p < .10$.

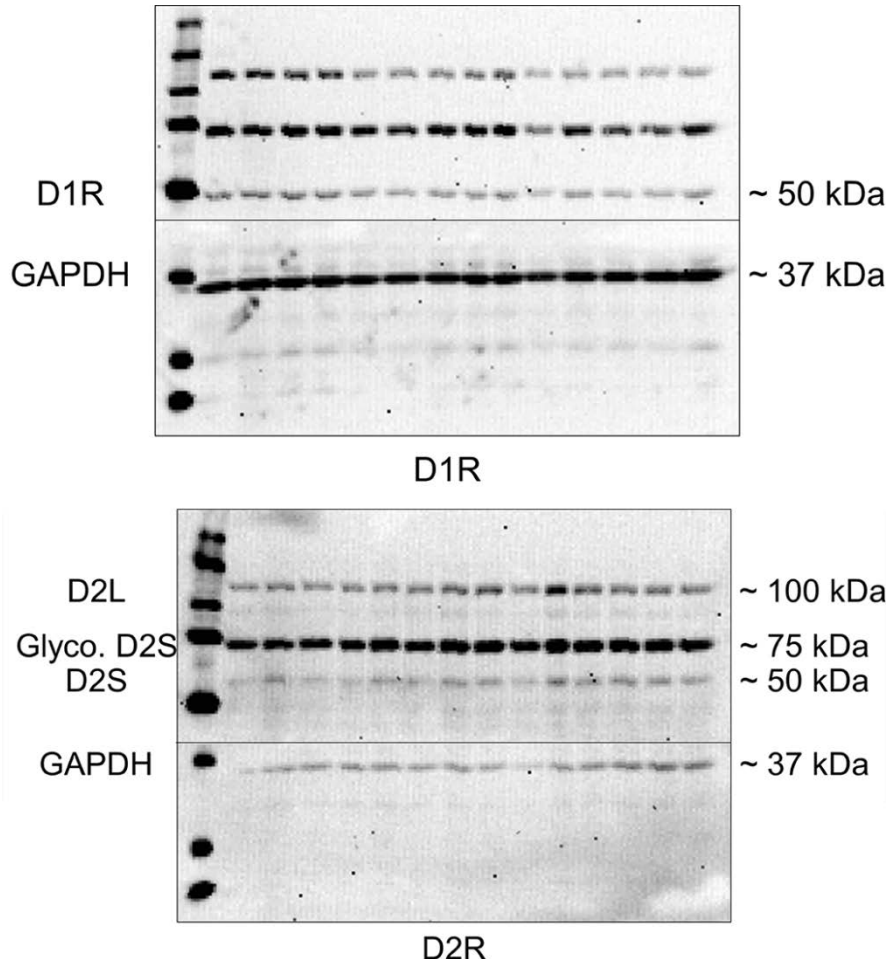

Figure 3. Western blot images for D1R (top) and D2R (bottom) protein bands from mPOA tissue of rats in the three hormonal priming groups. Bisected membranes are presented aligned for ease of viewing.

group, with a trend toward a significant increase compared to the O+O group. Finally there was a statistically significant and large overall effect of hormonal group on the 50kDa band ($F(2, 22) = 5.048$, $p = .016$, $\eta^2 = .315$. 90% CI [.040, .484]). Post hoc analyses revealed that EB+O protein levels were significantly increased compared to the EB+P group (Figure 4*A*).

In the PLC there was a trend towards a significant overall effect of hormonal group on D1R protein levels ($F(2, 25) = 3.234$, $p = .056$, $\eta^2 = .206$, 90% CI [.000, .377]), with a medium effect size. Hormonal manipulation had a statistically significant effect only on the 100kDa band relating to D2R protein levels ($F(2, 23) = 3.951$, $p = .033$, $\eta^2 = .256$, 90% CI [.012, .430]), also with a medium effect size. Post hoc analyses revealed that EB+O protein levels were significantly increased compared to the EB+P group. Neither the 75kDa band ($F(2, 23) = 0.151$, $p = .860$, $\eta^2 = .013$, 90% CI [.000, .099]) nor the 50kDa band ($F(2, 23) = 2.179$, $p = .136$, $\eta^2 = .159$, 90% CI [.000, .333]) were significantly different in terms of hormonal group, and showed a very small, and small to medium effect size, respectively (Figure 4*B*).

In the CP there was no statistically significant overall effect of hormonal group on D1R protein levels ($F(2, 25) = 0.814$, $p = .455$, $\eta^2 = .061$, 90% CI [.000, .201]) or on D2R protein levels at the 100kDa band ($F(2, 25) = 1.840$, $p = .180$, $\eta^2 = .128$, 90% CI [.000, .293]), the 75kDa band ($F(2, 25) = 1.843$, $p = .179$, $\eta^2 = .128$, 90% CI [.000, .294]), or the 50kDa band ($F(2, 25) = 0.480$, $p = .624$, $\eta^2 = .037$, 90% CI [.000, .155]). All effect sizes were small for this brain area (Figure 4*C*).

Log-transformed D1R/D2R ratios were calculated for each of the three D2R protein bands for the brain areas of interest. A statistically significant and large effect of hormonal group was found for each ratio in the mPOA: D1R/D2R(100kDa) ($F(2, 18) = 10.004$, $p = .001$, $\eta^2 = .526$, 90% CI [.187, .660]), D1R/D2R(75kDa) ($F(2, 18) = 6.105$, $p = .009$, $\eta^2 = .404$, 90% CI [.074, .567]), and D1R/D2R(50kDa) ($F(2, 18) = 7.555$, $p = .004$, $\eta^2 = .456$, 90% CI [.117, .607]). Post hoc analyses indicate that in each case the EB+O group had a significantly decreased D1R/D2R ratio than the EB+P group, again indicating that with only EB on board the DAR ratio is tilted towards D2R, and adding P reverses the pattern (Figure 4*D*).

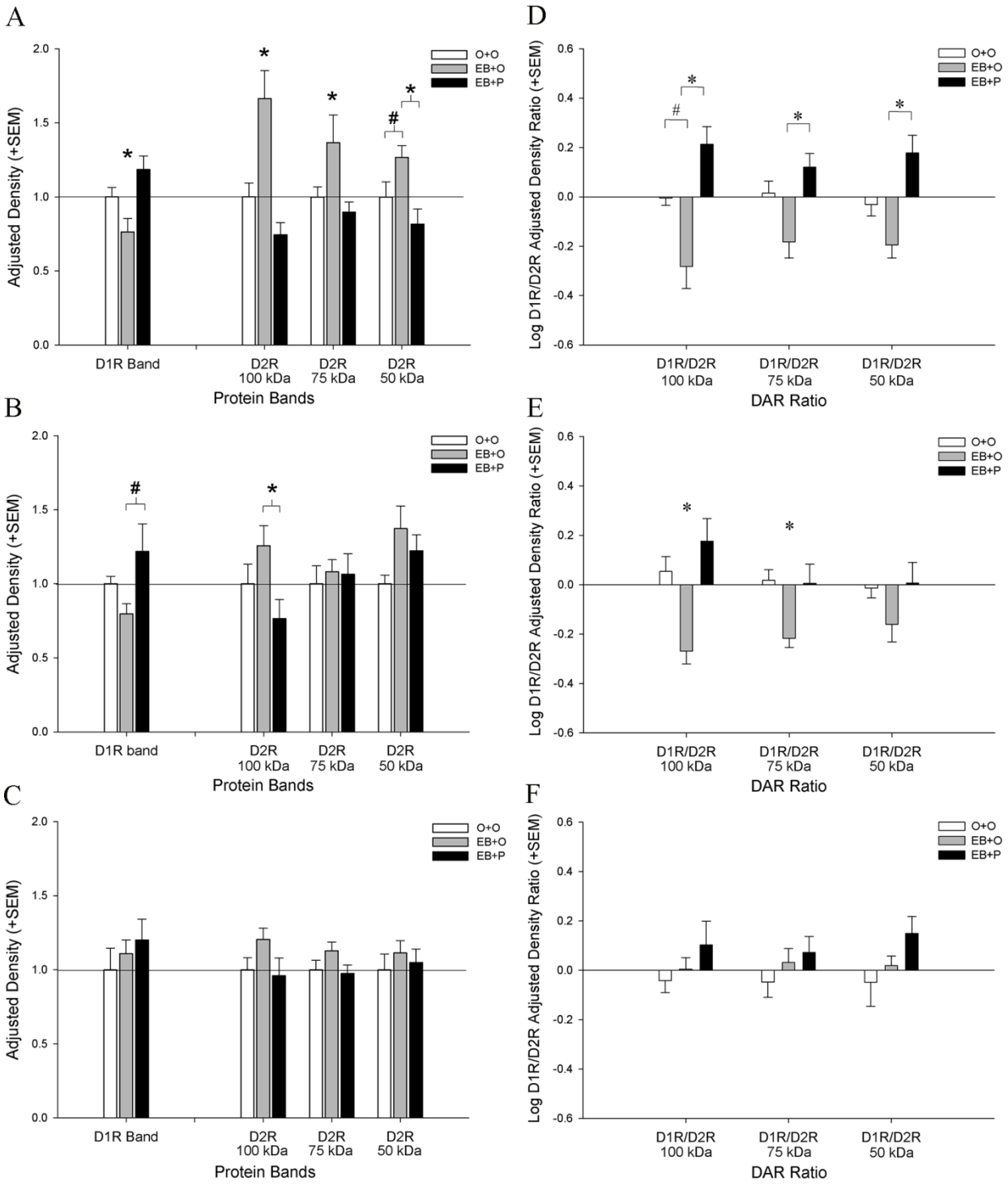

Figure 4. Western blot analyses of hormonal group on standardized protein density of the DAR subtypes. Each value represents the mean standardized protein level respective to the control (O+O) group. Error bars represent the SEM. (A) DAR protein levels in the mPOA. Females primed with EB+O show a decrease in D1R protein levels compared with females primed with

EB+P. Females primed with EB+O also show an increase in D2R protein levels at the 100 kDa band compared with the other two groups. The EB+O group shows an increase in D2R protein levels at the 75 kDa band compared with females primed with EB+P, and a trend towards an increase compared with the control group (O+O). The EB+O group also shows an increase in D2R protein levels at the 50 kDa band compared with the EB+P group. (B) DAR protein levels in the PLC. No effect of hormonal manipulation was found on D1R protein levels in the PLC. For D2R, only the 100 and 75 kDa bands showed a statistically significant effect, in both cases, females treated with EB+O showing an increase compared with the EB+P group. D2R protein levels at the 50 kDa band did not show any significant difference between hormonal groups. (C) DAR protein levels in the CP. There were no effects of hormonal groups on D1R or D2R protein levels. (D) Log-transformed D1R/D2R protein level ratio in the mPOA. Females in the EB+O group had a decreased D1R/D2R protein level ratio compared with females in the EB+P group at each band of D2R. (E) Log-transformed D1R/D2R protein level ratio in the PLC. The EB+O group had a lower D1R/D2R protein level ratio compared with the other two groups only at the 100 kDa band. The other two bands showed no effect of hormonal group on the D1R/D2R protein level ratio. (F) Log-transformed D1R/D2R protein level ratio in the CP. No effects of hormonal group were found for any of the D1R/D2R protein level ratios across any of the three D2R bands. * $p < .05$; # $p < .10$.

In the PLC, a statistically significant and large effect of hormonal group was found for the log-transformed D1/D2 ratio only for the 100kDa ($F(2, 23) = 10.911$, $p < .001$, $\eta^2 = .487$, 90% CI [.191, .622]) and 75kDa bands ($F(2, 23) = 5.577$, $p = .011$, $\eta^2 = .327$, 90% CI [.053, .492]). In both cases, post hoc analyses revealed that the EB+O group had a significantly reduced D1R/D2R ratio compared to the two other groups. No statistically significant main effect of hormonal group was found for the log-transformed D1R/D2R ratio of the 50kDa band ($F(2, 23) = 1.733$, $p = .199$, $\eta^2 = .131$, 90% CI [.000, .301]), and the effect size was small to medium (Figure 4*E*).

For the CP, no statistically significant main effect of hormonal groups were found for log-transformed D1R/D2R ratios for the 100kDa ($F(2, 25) = 1.097$, $p = .349$, $\eta^2 = .081$, 90% CI [.000, .231]), 75kDa ($F(2, 25) = 0.992$, $p = .385$, $\eta^2 = .074$, 90% CI [.000, .221]), or 50kDa ($F(2, 11) = 2.122$, $p = .166$, $\eta^2 = .278$, 90% CI [.000, .485]) protein bands. Small effect sizes were found for the ratios involving the 100 and 75kDa bands, and a medium to large effect size was found for the ratio involving the 50kDa band (Figure 4*F*).

Autoradiography

Representative images of DAR functional binding and examples of the brain series for the mPOA/CP and PLC are seen in Figure 5. A trend towards a significant three-way interaction was found between DAR subtype, brain series, and hormonal group ($F(4, 30) = 2.566$, $p = .058$, $\eta_p^2 = .255$, 90% CI [.000, .374]), with a medium to large effect size, in the mPOA. Simple effects analyses indicated that increased D2R binding levels in the EB+P group at the third series are producing this result. A statistically significant interaction with a medium to large effect size was found between DAR subtype and brain series ($F(2, 30) = 4.870$, $p = .015$, $\eta_p^2 = .245$, 90% CI [.031, .405]), with simple effects analyses indicating that D1R binding levels were significantly decreased compared to D2R binding levels at the third brain series. Similarly, D1R binding levels overall were close to being significantly lower than D2R binding levels ($F(1, 15) = 3.929$, $p = .066$, $\eta_p^2 = .208$, 90% CI [.000, .446]), and showed a medium effect size, indicating the possibility that increased power would result in a statistically significant difference. No statistically significant interactions were found between DAR subtype and hormone group ($F(2, 15) = 0.978$, $p = .399$, $\eta_p^2 = .115$, 90% CI [.000, .306]), or brain series and hormone group ($F(4, 30) = 2.062$, $p = .111$, $\eta_p^2 = .216$, 90% CI [.000, .333]), and no significant main effects were

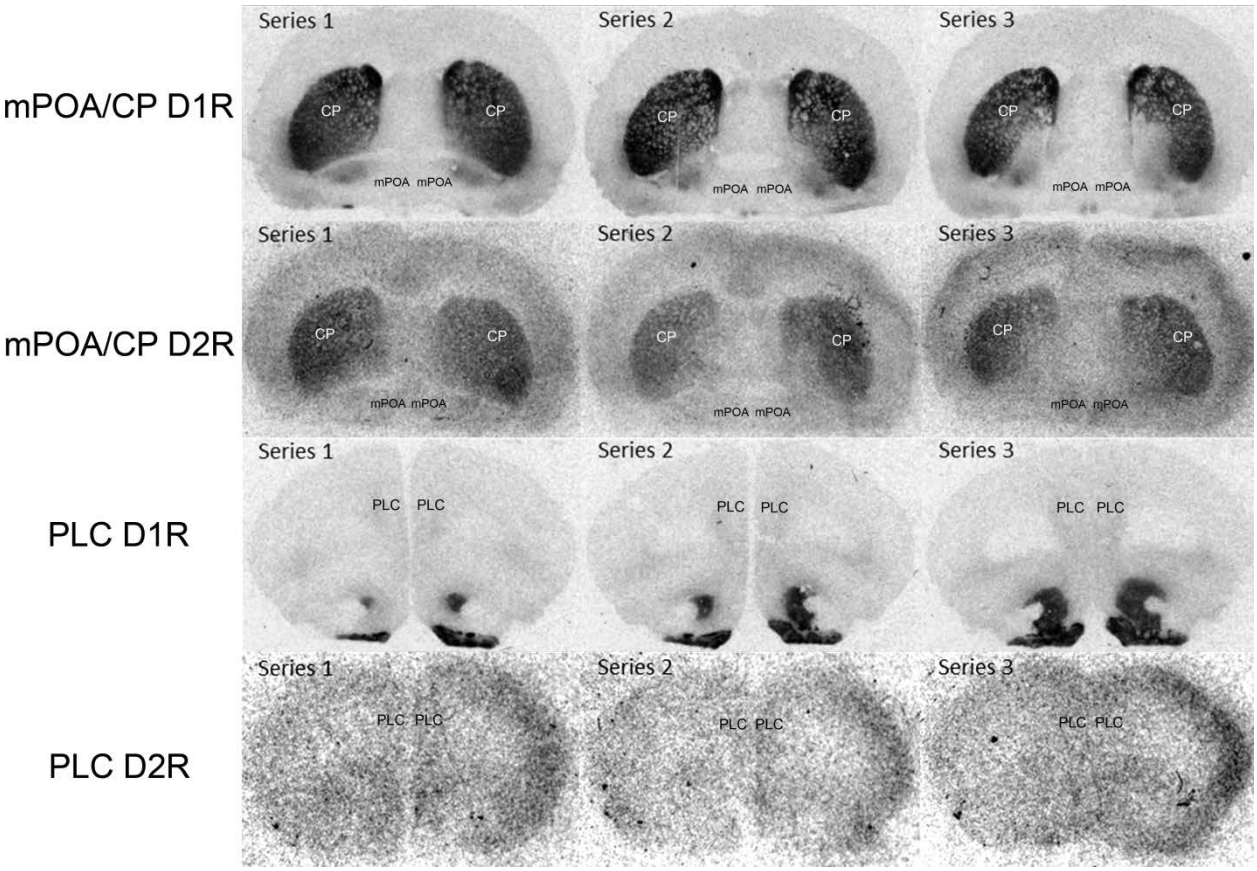

Figure 5. Autoradiographic film images for the three brain series taken for each area. The top two rows depict D1R and D2R binding in the mPOA/CP, and the bottom two rows depict D1R and D2R binding in the PLC. Each series corresponds to nine sequential slices taken across each area.

found for brain series ($F(2, 30) = 0.644$, $p = .532$, $\eta_p^2 = .041$, 90% CI [.000, .157]) or hormone group ($F(2, 15) = 1.348$, $p = .290$, $\eta_p^2 = .152$, 90% CI [.000, .350]). All effect sizes ranged from small to medium (Figure 6*A*).

Results of functional binding in the PLC found no statistically significant results. There were no statistically significant interactions between DAR subtype, brain series, and hormone group ($F(4, 22) = 0.140$, $p = .965$, $\eta_p^2 = .025$, 90% CI [.000, .016]), or between DAR subtype and brain series ($F(2, 22) = 0.083$, $p = .921$, $\eta_p^2 = .007$, 90% CI [.000, .079]), both of which resulted in very small effect sizes. Similarly, there was not a statistically significant interaction between DAR subtype and hormone group ($F(2, 11) = 1.507$, $p = .264$, $\eta_p^2 = .215$, 90% CI [.000, .429]), nor between brain series and hormone group ($F(2.564, 14.102) = 0.692$, $p = .551$, $\eta_p^2 = .112$, 90% CI [.000, .272]), but in both those interactions the effect size was in the medium range. Finally, there were no statistically significant main effects found for DAR subtype ($F(1, 11) = 3.091$, $p = .106$, $\eta_p^2 = .219$, 90% CI [.000, .482]), brain series ($F(1.282, 14.102) = 0.075$, $p = .846$, $\eta_p^2 = .007$, 90% CI [.000, .149]), or hormone group ($F(2, 15) = 1.348$, $p = .290$, $\eta_p^2 = .152$, 90% CI [.000, .350]), again with all effect sizes ranging from very small to medium in nature (Figure 6*B*).

Similar to the PLC, the CP found no statistically significant findings for binding density differences. There were no statistically significant interactions between DAR subtype, brain series, and hormone group ($F(4, 30) = 0.687$, $p = .607$, $\eta_p^2 = .084$, 90% CI [.000, .161]), between DAR subtype and brain series ($F(2, 30) = 0.729$, $p = .491$, $\eta_p^2 = .046$, 90% CI [.000, .166]), between DAR subtype and hormone group ($F(2, 15) = 0.577$, $p = .574$, $\eta_p^2 = .071$, 90% CI [.000, .243]), nor between brain series and hormone group ($F(4, 30) = 0.591$, $p = .672$, $\eta_p^2 = .073$, 90% CI [.000, .141]), with all interaction effect sizes being very small. Additionally, there were no statistically significant main effects found for DAR subtype ($F(1, 15) = 2.182$, $p = .160$, $\eta_p^2 = .127$, 90% CI [.000, .370]), brain series ($F(2, 30) = 2.191$, $p = .129$, $\eta_p^2 = .127$, 90% CI [.000, .282]), or hormone group ($F(2, 15) = 1.326$, $p = .295$, $\eta_p^2 = .150$, 90% CI [.000, .347]), with all effect sizes being in the small to medium range (Figure 6*C*).

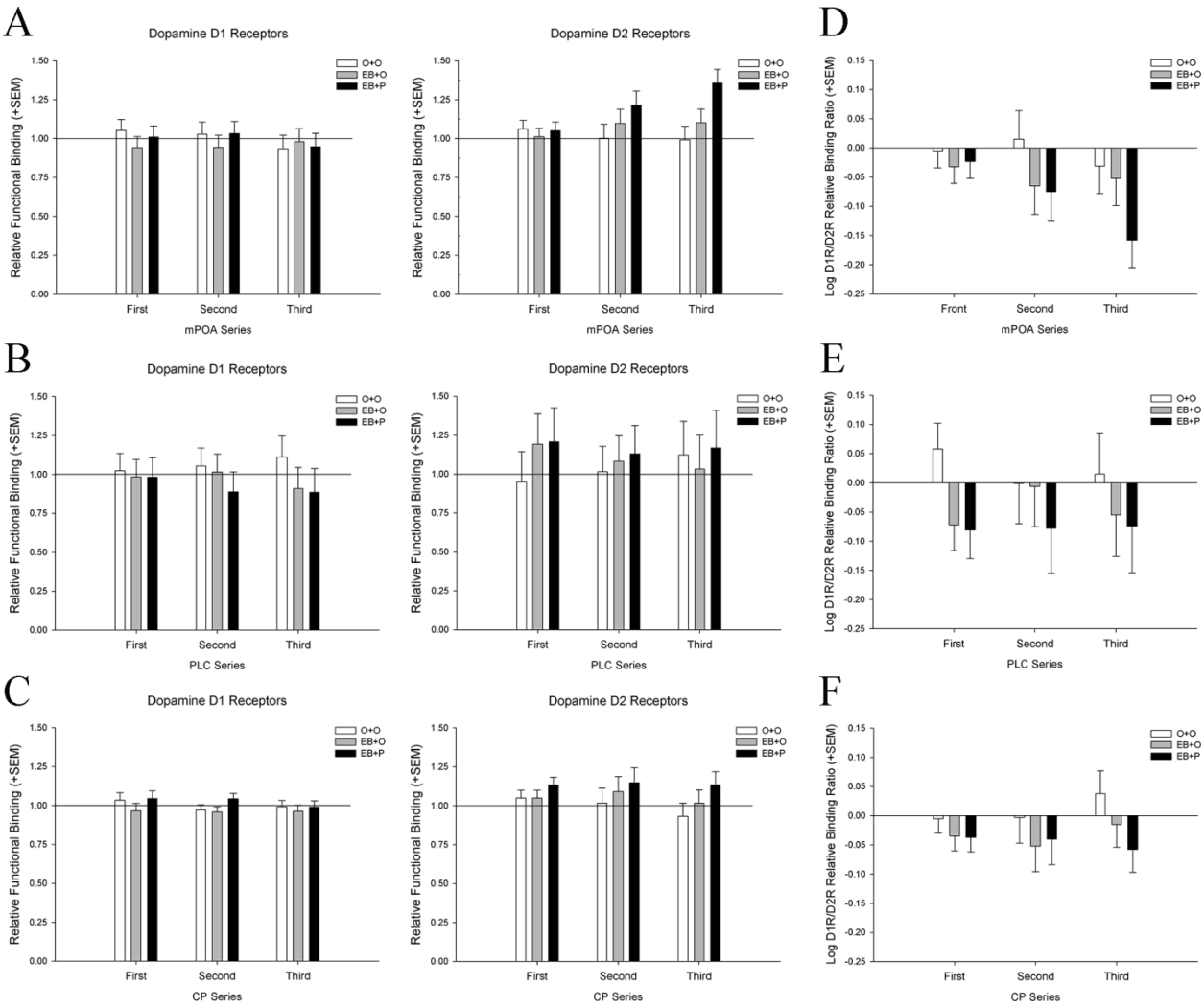

Figure 6. Autoradiography analyses of hormonal group on standardized functional binding densities of the DAR subtypes. (A) DAR functional binding levels in the mPOA. A trend towards a three-way interaction was found between DAR subtype, brain series, and hormonal group, driven by increased D2R binding levels in the third series of the mPOA in females primed with EB+P. There was also a significant interaction between DAR subtype and brain series, with decreased D1R binding levels compared with D2R binding levels in the third series of the mPOA. No other significant interactions or main effects were found. (B) DAR functional binding levels in the PLC. No effects of hormonal groups on DAR functional binding levels were found. (C) DAR functional binding levels in the CP. No effects of hormonal groups on DAR functional binding levels were found. (D) Log-transformed D1R/D2R functional binding ratio in the mPOA. There was a trend towards a significant interaction between brain series and hormonal group, driven by the EB+P group having a decreased D1R/D2R functional binding

ratio in the third brain series of the mPOA. A significant main effect of brain series was also found, with the third series having a lower D1/D2R functional binding compared with the first series. No other significant main effects were found. (E) Log-transformed D1R/D2R functional binding ratio in the PLC. No significant interactions or main effects were found for any of the D1R/D2R functional binding ratios. (F) Log-transformed D1R/D2R functional binding ratio in the CP. No significant interactions or main effects were found for any of the D1R/D2R functional binding ratios.

Log-transformed D1R/D2R ratios were calculated for each of the three areas of interest and compared across the three brain slice sequences and between hormonal groups. In the mPOA there was a trend towards a significant interaction between brain series and hormone group (F(4, 30) = 2.253, p = .087, η_p^2 = .231, 90% CI [.000, .349]), with a medium effect size, driven by the EB+P group having a decreased ratio in the third brain series. There was also a statistically significant main effect of brain series (F(2, 30) = 4.869, p = .015, η_p^2 = .245, 90% CI [.031, .405]), also with a medium effect size. Bonferroni post hoc analyses indicated the third series was significantly less than the first. There was, however, no statistically significant main effect of hormone group (F(2, 15) = 1.061, p = .371, η_p^2 = .124, 90% CI [.000, .317]), and the effect size was small (Figure 6*D*).

No statistically significant results were found for log-transformed D1R/D2R ratios in the PFC, including the interaction between brain series and hormone group (F(4, 22) = 0.339, p = .849, η_p^2 = .058, 90% CI [.000, .112]), and the main effects of brain series (F(2, 22) = 0.024, p = .977, η_p^2 = .002, 90% CI [.000, .040]) and hormone group (F(2, 11) = 1.261, p = .321, η_p^2 = .187, 90% CI [.000, .401]) (Figure 6*E*). Similarly, the log-transformed D1R/D2R ratios in the CP produced no significant interaction between brain series and hormone group (F(4, 30) = 0.839, p = .511, η_p^2 = .101, 90% CI [.000, .188]), nor significant main effects of brain series (F(2, 30) = 0.724, p = .493, η_p^2 = .046, 90% CI [.000, .166]) or hormone group (F(2, 15) = 1.061, p = .371, η_p^2 = .124, 90% CI [.000, .317]). All effect sizes related to the log-transformed D1R/D2R ratios in these two areas were in the very small to medium range, and were smaller than the effects found in the mPOA (Figure 6*F*).

Discussion

The data from these three experiments indicate that administration of ovarian steroids alters the levels of the two DAR subtypes in the mPOA of female rats, creating a DAR signature that facilitates appetitive sexual motivation and behaviour. Using immunohistochemistry to count cells stained for DAR, Western blots to examine protein levels, and autoradiography to measure functional binding densities, evidence for changes in D1R and D2R have been found for three different hormonal conditions. Specifically, immunohistochemical and Western blot analysis collectively demonstrate that females primed only with estradiol benzoate (EB+O) show a decrease in D1R levels and an increase in D2R levels in the mPOA compared to females

primed only with oil (O+O) or primed with both EB and progesterone (EB+P). Similar trends are found for alterations in DAR levels in the PLC under those hormonal conditions, though the evidence is not as clear. There were no changes in DAR levels in the CP across the three hormonal conditions.

The results from the autoradiography experiment indicated no effect on binding density in the PLC or CP but in contrast with the two other studies found that females primed with EB+P showed an increase in D2R binding density in areas of the third sequence of slices obtained from the mPOA, which corresponds to the middle coronal sections of this structure that spans from approximately -0.48mm to -0.72mm from Bregma (corresponding to panels 37-39 from Paxinos & Watson, 2005). This section represents the portion of the mPOA where previous behavioural studies from our lab have aimed cannulae infusers for drug administration (e.g., Pfaus et al., 2007; Graham & Pfaus, 2010; Graham & Pfaus, 2012). The lack of effects of hormonal priming in the more anterior areas of the mPOA that we sampled indicate that the mPOA is not a homologous structure when it comes to D2R functional binding, and that coronally the central portion of the mPOA is most sensitive to changes in D2R after EB+P priming. Further investigation into this is warranted.

The autoradiography results indicating an increase in D2R functional binding in a certain portion of the mPOA after EB+P priming seem to contradict those of the immunohistochemistry and Western blot experiments. However, if considered similar to the results of PET studies (Laruelle, D'Souza, Baldwin, Abi-Dargham, Kanes, Fingado, Seibyl, Zoghbi, Bowers, Jatlow, Charney, & Innis, 1997a), it is likely that an increase in D2R functional binding reflects less competitive binding by endogenous DA as a function of hormonal priming. Thus, the increase in D2R binding by the radioligand is reflective of less D2R binding by DA during EB+P conditions, in line with the hypotheses that EB+P animals have a D1R-tilted system. Consistent with this, endogenous DA has been found to affect binding levels in previous autoradiography studies examining DA D3 receptors (Schotte, Janssen, Bonaventure, & Leysen, 1996). It should be noted that pre-incubations may prevent endogenous DA from affecting D2R binding, though in previous cases the radioligand used was not [^{3}H]Sulpiride (e.g., Schotte et al., 1996; Laruelle et al., 1997a).

If an increase in D2R functional binding seen in the EB+P group does indeed reflect unbound D2R, then results of the log-transformed D1R/D2R ratios for each of the three

experiments are in agreement with previous behavioural findings that different hormonal treatments of female rats modifies the function and/or role of DAR in the mPOA. Dopamine in the mPOA has been shown to be important in the control of appetitive sexual behaviours in female rats (Graham & Pfaus, 2010; Graham & Pfaus, 2012). Similar to their role in the sexual behaviour of the male rat (for review, see Hull & Dominguez, 2007), DAR subtypes play opposing roles on female appetitive sexual behaviour. Specific to females however, the exact role of the two DAR subtypes may depend on the hormonal profile of the female. In females primed only with EB (EB+O), D2R facilitate appetitive behaviours and D1R inhibit them. The addition of progesterone, in EB+P females, results in the opposite pattern as D1R stimulation promotes appetitive behaviours and D2R activation decreases them (Graham & Pfaus, 2010; Graham & Pfaus, 2012). The current studies provide insight into the mechanism responsible for this switch in DAR roles.

Administration of EB to females results in a decrease in D1R protein, but an increase in D2R protein, in the mPOA as measured by the Western blots. The addition of P not only restores these levels to those seen in females when no ovarian steroid hormones are present, but surpasses levels of the O+O group, though not statistically significant. Adding P to EB also results in an increased amount of D2R free to bind, as measured by autoradiography. This could be due either to an upregulation of D2R, or a decrease in receptor occupancy by endogenous DA. In either case, it would appear that alteration of these receptor levels (or their availabilities) by the steroid hormones may be partly responsible for the increase in motivation to engage in sexual activity that accompanies a female that is fully sexually receptive. Producing either more D2R or less competition from endogenous DA, via administration of EB+O, may spur female rats to engage in sexual activity without being fully sexually-primed. Although this is likely an artificial laboratory phenomenon, this dual control of DAR under EB+O conditions could be one of several mechanism(s) underlying the onset of menopause, or the decrease in libido seen at that time, as a result of steroid hormone level fluctuation.

A related explanation may be that the D1R/D2R ratio is the driving force in the control of appetitive female sexual behaviour, rather than the overall amount of receptor available. Compared to the baseline seen in the O+O group, the EB+O group has higher levels of available D2R than D1R, resulting in a low D1R/D2R ratio. This ratio is reversed with the addition of P, so that there is more D1R available compared to D2R and a higher D1R/D2R ratio in the EB+P

group. A high D1R/D2R ratio might be important for increasing sexual motivation, resulting in the display of appetitive behaviours. Females in both the O+O and EB+O group, which do not have more D1R relative to D2R, do not exhibit these behaviours often. Consummatory behaviours meanwhile are under the control of other brain areas, namely the VMH (Flanagan-Cato, 2011) and the VTA (Frye, 2011).

Collectively the data from the current studies provide neurophysiological evidence of a mechanism underlying the critical role for DAR in the display of female appetitive sexual behaviour, especially in the mPOA, and that ovarian steroid hormones affect this mechanism. The ratio of DARs have been found to be altered in a wide variety of circumstances, including aging (Seeman, Bzowej, Guan, Bergeron, Becker, Reynolds, Bird, Riederer, Jellinger, Watanabe, & Tourtellotte, 1987), circling behaviour (Glick, Lyon, Hinds, Sowek, & Titeler, 1988; Roy, Buyer, & Licari, 1990), locomotion (Spencer, Torres-Altoro, Falcon, Arey, Marvin, Goldberg, Bibb, & McClung, 2012); pain (Hagelberg, Forssell, Aalto, Rinne, Scheinin, Taiminen, Nagren, Eskola, & Jaaskelainen, 2003a; Hagelberg, Forssell, Rinne, Scheinin, Taiminen, Aalto, Luutonen, Nagren, & Jaaskelainen, 2003b), dyskinesia (Gagnon, Gomez-Mancilla, Bedard, & Di Paolo, 1993), and schizophrenia (Hess, Bracha, Kleinman, & Creese, 1987; Winterer & Weinberger, 2004; but also see Hirvonen, van Erp, Huttunen, Nagren, Huttunen, Aalto, Lonnqvist, Kaprio, Cannon, & Hietala, 2006). DAR ratios have been speculated to be involved in male sexual behaviour for a variety of animals (see Kleitz, Cornil, Balthazart, & Ball, 2009). Regarding female sexual behaviour, the D1R/D2R ratio in the mPOA may be part of a larger circuitry in the control of appetitive responses. This specifically may include the nucleus accumbens (NAc) and striatum, as Becker (1999) has posited that estrogenic modulation on dopamine in these areas enhance specific components of pacing behaviour. The mPOA could indirectly communicate with these areas as it sends efferents to the VTA (Simerly & Swanson, 1988), which in turn has DAergic neurons projections to the NAc and striatum (Swanson, 1982; Deutch, Goldstein, Baldino, & Roth, 1988). In this manner, estrogen may be having dual effects on two separate, yet connected, DA systems in order to enhance female reproductive behaviours. Dopamine release during copulation in the mPOA and NAc is identical for both male (Blackburn et al., 1992) and female rats (Pfaus et al., 1995; Matuszewich et al., 2000). In fact, hormonal administration alone is sufficient to increase DA release in the mPOA of females, but only when P is co-administered with a low dose of EB (Matuszewich et al., 2000).

Minimal information is currently known about the mechanism by which P may alter DAR, though evidence has been found demonstrating that P interacts with DA in other brain areas important for female sexual behaviour. For example, P has been found to increase DA levels and D1R density in the striatum (Dluzen & Ramirez, 1989; Levesque & Di Paolo, 1990), and it has also been shown to increase DA levels in the VTA (Frye et al., 2000). In the CP, P decreases D2R four hours after independent administration, an effect not seen when EB is given prior. However there was an increase in D2R levels 24 hours after EB+P treatment (Fernandez-Ruiz et al., 1989).

Taken together, the results of the current study show estradiol and progesterone coordinate the activation of D2R and D1R in a manner that leads to a critical ratio of activation for appetitive female sexual response. Rats primed with EB+O have a low D1R/D2R ratio, and display low-levels of lordosis, requiring additional neurotransmission (e.g., augmented D2R binding and/or other neurochemical system activation) to augment appetitive sexual behaviours. The addition of P results in a switch to a higher D1R/D2R ratio, and results in a display of high levels of appetitive sexual behaviours. This behavioural output is likely due to an interaction of P and D1R pathways, possibly originating in the mPOA and communicating with areas responsible for lordosis, such as the VMH and/or VTA, in order to time stereotypical female copulatory bouts and/or augment the impact of sexual stimulation.

CHAPTER 4: STEROID HORMONES MODULATE DOPAMINE RECEPTOR SUBTYPES ON GLUTAMATE AND GABA NEURONS IN THE MEDIAL PREOPTIC AREA OF FEMALE RATS THAT PROJECT TO THE VENTROMEDIAL HYPOTHALAMUS AND VENTRAL TEGMENTAL AREA: A TRIPLE-LABELLING STUDY

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Abstract

Dopamine receptor (DAR) activation in the medial preoptic area (mPOA) is critical for appetitive sexual behaviours in the female rat. In females primed with estradiol benzoate (EB), activation of D2 receptors (D2R) in the mPOA increases appetitive behaviours, whereas in females primed with both EB and progesterone (EB+P) these behaviours increase after D1 receptor (D1R) activation. Here we used fluorescence immunohistochemistry to map pathways projecting from the mPOA to either the VMH or the VTA, regions that regulate female sexual behaviour. Ovariectomized rats were infused with the retrograde tracer Fluoro-Gold (FG) into the VMH or VTA, and were then assigned randomly to one of three steroid treatment groups: EB+P (10μg and 500μg, respectively), EB alone, or the oil vehicle, with hormones administered 48h and 4h prior to euthanization. Immunofluorescence was conducted on mPOA slices to label glutamate or GABA neurons, and neurons expressing D1R or D2R. Thus, we quantified FG-positive cells, neuronal type, DAR expression, and whether the neurons project from the mPOA to either the VMH or VTA. EB alone increased the number and proportion of GABA cells projecting to the VMH. Administration of EB+P increased the proportion of glutamate cells containing D2R projecting to the VMH. Administration of EB (with or without P) decreased the proportion of GABA cells containing D1R projecting to the VTA. These data show that ovarian steroids modulate the expression of DAR in the mPOA that differentially regulate solicitations and lordosis via their projections to the VTA and VMH.

Keywords: Ovarian Hormones; Dopamine Receptors; Glutamate; GABA; Fluoro-Gold;

Introduction

The ovarian steroids estradiol (E2) and progesterone (P) are critical for the full expression of appetitive solicitations and lordosis behaviour in the female rat (Pfaff, 1999; Pfaus, Jones, Flanagan-Cato, & Blaustein, 2015). Dopamine (DA) actions within the medial preoptic area (mPOA) and mesolimbic DA terminal regions are also critical for the display of appetitive solicitations, whereas lordosis is facilitated by DA actions in the VMH. Coordination of these responses in time allows the female to pace the clitoral and cervical stimulation she receives from the male (Pfaus et al., 1999; Pfaus, 2009; Parada et al., 2010). Estradiol benzoate (EB), alone or in combination with P, alters the ratio of DA receptor (DAR) subtypes in the mPOA to promote solicitations or lordosis (Graham & Pfaus, 2010; Graham & Pfaus, 2012; Graham, Gardner Gregory, Hussain, Brake, & Pfaus, 2015). Females primed with EB alone have a low D1R/D2R ratio and display low levels of lordosis, whereas females primed with both EB+P have an elevated D1R/D2R ratio and high levels of appetitive sexual behaviours. The increase in solicitations is likely due to an interaction of P and D1R in the mPOA that alters input to regions like the VMH and ventral tegmental area (VTA) (Pfaus et al., 2015).

Glutamate (GLU) in the VMH normally inhibits lordosis (reviewed in Pfaus et al., 2015), and EB treatment decreases extracellular concentrations of GLU (Luine et al., 1997; Georgescu et al., 2014). In addition, activation of GLU neurons decreases following EB treatment, with or without P (Georgescu et al., 2009). Infusions of ionotropic GLU receptor agonists to the VMH inhibit solicitations and lordosis, whereas antagonists of these receptors facilitate both aspects of female sexual behaviour (Georgescu & Pfaus, 2006a; Georgescu & Pfaus, 2006b). In contrast, EB increases the synthesis of γ-aminobutyric acid (GABA) in the VMH, and inhibition of GABA synthesis in the VMH inhibits female sexual behaviours (McCarthy, 1995; Luine et al., 1999) thus making GABA actions there disinhibitory. In the VTA, potential membrane actions of P at GABA-A receptors facilitate the intensity of lordosis (Frye, 2001; Frye, 2011) as few intracellular progesterone receptors exist in this region (MacLusky & McEwen, 1980; Blaustein, Tetel, Ricciardi, Delville, & Turcotte, 1994). Alterations of D1R in the VTA mediate P-facilitated lordosis (Frye, Walf, & Sumida, 2004). D1R have also been found on GABA terminals in the VTA (Bayer & Pickel, 1991), suggesting a mechanism by which DA facilitates GABA release. Although mesolimbic DA cell bodies in the VTA are normally inhibited by GABA interneurons, GABA projections to GABA interneurons can disinhibit DA cell bodies

and lead to activation of mesolimbic DA release, as is observed in female rats during paced copulation (Yoest, Cummings, & Becker, 2014).

Does the D1R/D2R ratio set up by E2 and P in the mPOA alter appetitive and consummatory female sexual behaviours by modulating the relative influence of GABA and GLU projections to the VMH and VTA? The purpose of the present experiment was to determine whether D1R and/or D2R were expressed on GLU or GABA projections to the VMH or VTA as a function of steroid hormone treatment.

Materials and Methods

Animals and surgery

Adult female Long-Evans rats, weighing approximately 250g were obtained from Charles River Canada, Inc. (St.-Constant, QC). Animals were pair-housed in Plexiglas cages with wood chip bedding, and allowed ad libitum access to water and regular rat chow. All rats were housed in the same colony room with the temperature kept constant at 21ºC, on a reversed 12-hour light/dark cycle, with lights off at 0800.

Animals were acclimated to the colony for one week, after which they underwent bilateral ovariectomy via a lumbar incision allowing for hormonal manipulation and control. Ovariectomies were performed under anaesthesia consisting of ketamine hydrochloride (100mg/ml) and xylazine hydrochloride (20mg/ml) mixed in a 4:3 ratio, respectively, at a dose of 1ml/kg administered ip. One week of recovery time was provided following the surgery. All animal procedures conformed to the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Sexual behaviour training

Rats were given sexual experience in order to see how hormonal manipulation affected neuronal projections in an experienced animal, allowing for the replication and extension of previous knowledge from findings in our lab (i.e., Graham et al., 2015). Sexual experience was provided through a series of four 30min trials with a male in a bilevel chamber, spaced four days apart. The male was placed in the chamber for a 5-min acclimation period, after which the sexually receptive female entered. Experimenter observation assured that at least one ejaculation occurred during each trial. Females were hormonally primed prior to the trial to ensure sexually receptivity with a two-part combination of subcutaneous hormone injections consisting of estradiol benzoate (1,3,5(10)-Estratriene-3,17b-diol 3-benzoate; Cat# 50-50-0) and progesterone

(4-Pregnene-3,20-dione; Cat# 57-83-0), both of which were obtained from Steraloids (Newport, RI, USA). Estradiol benzoate (10µg in 0.1ml of sesame oil) and progesterone (500µg in 0.1ml of sesame oil) were administered 48h and 4h prior to each sexual bout, respectively. Following the fourth sexual trial, females were given a 3-week hormone washout period prior to Fluoro-Gold infusion.

Fluoro-Gold infusion

To determine anatomical connections projecting from the mPOA to the VMH or VTA, the retrograde tracer Fluoro-Gold (FG; Fluorochrome, LLC; Denver, CO, USA) was infused into the VMH (AP -2.64, ML +0.6, DV -8.0 mm from Bregma, incision bar set at 0; Paxinos & Watson, 2005) or the VTA (AP -6.24, ML +0.7, D -7.2 mm from Bregma, incision bar set at 0; Paxinos & Watson, 2005). Rats were anaesthetized with the ketamine/xylazine mixture, as above, and a 21-gauge guide cannula was lowered into place 1 mm above the target area, using a Kopf stereotaxic instrument. An infusion cannula, modified from a 27-gauge needle and cut 1mm longer than the guide cannula, was lowered to administer a 2% FG solution (dissolved in saline) at a rate of 0.2µl/min for 1min using an infusion pump (Harvard Apparatus, Pump 22). The infusion cannula was left in place for 10min to allow for absorption. Following this, animals were left undisturbed until hormonal injections were made.

Hormonal manipulation

Eight days after the FG infusion, and 48h before euthanasia, rats received a subcutaneous injection of either EB (10µg in 0.1ml of sesame oil) or the oil vehicle control (0.1ml of sesame oil). Two days later, and 4 hours before euthanasia, on the 10th day following FG infusion, rats received a second subcutaneous injection of either P (500µg in 0.1ml of sesame oil) or the vehicle control. The combination of the two injections resulted in female rats being randomly assigned to one of three hormonal groups (n = 5 for each group): an oil control group (O+O), a partially-primed EB alone group (EB+O), and a fully-primed group (EB+P). The timing of the injections was controlled to mimic that of hormonal priming commonly used to prime appetitive and consummatory sexual behaviour (Pfaus et al., 2015).

Intracardial perfusion and histology

Four hours after their second hormone injection, rats received an overdose of sodium pentobarbital (120mg/kg, i.p.), and were perfused intracardially with 250ml of ice-cold phosphate-buffered saline and 4% paraformaldehyde made in 0.1M phosphate buffer (PB).

Brains were removed, postfixed for 4h in 4% paraformaldehyde in 0.1M PB, and transferred to a 30% sucrose solution for approximately 48h, stored at 4ºC. Brains were then flash frozen on dry ice and stored at -80ºC until slicing. Coronal slicing was done at 35 microns in a set of three series. Each series was transferred to cryoprotectant and stored at 20ºC until fluorescent immunohistochemistry (FIHC).

Placements were verified using a fluorescent microscope examining FG diffusion, accompanied by infuser cannula tract when possible. The a priori criterion set allowed for brains that displayed the infuser cannula inside the target area, or if no tract was present, a meaningful amount of FG visible within target area, were included in statistical analyses. Placement data and examples are shown in Figure 1.

Fluorescent immunohistochemistry (FIHC)

FIHC was conducted examining two different projection sites (VMH or VTA), two different neurotransmitters (GABA or GLU) and two different DA receptors (D1R or D2R). Thus, the combinations of FIHC runs allowed us to observe if DAR are located on neurons stained for either GABA or GLU within a system connecting the mPOA to either the VMH or VTA, using a procedure adapted from one described previously (Tobiansky et al., 2013). The retrograde tracer FG, used to determine efferent projections from the mPOA to either the VMH or VTA, has autofluorescent properties with an emission maximum of 461nm, visualized as a blue wavelength. Before FIHC procedures to visualize the other antigens, all sections were first rinsed once for 1min, followed by 6 times of 5min each, in order to remove the cryoprotectant. All steps were done at room temperature on a rotator.

The procedure for visualizing the neurotransmitter GLU started with a 1h incubation in a blocking solution (BS) consisting of 2% normal goat serum (NGS; Vector Laboratories, Cat# S-1000) and 0.4% Triton-X (Fisher Scientific, A00062) in 0.1M PB. The sections were then incubated overnight in BS with a primary antibody recognizing L-glutamic acid (GLU) raised in mouse (1:10,000; Sigma-Aldrich, Cat# G9282). The following day, sections were rinsed once for 1min, followed by 3 rinses of 5min each, and incubated with biotinylated goat anti-mouse IgG (1:200; Vector Laboratories, Cat# BA-9200) in BS for 1h. Sections were rinsed, and incubated with an avidin-biotin complex (1:1000; ABC-elite, Vector Laboratories, Cat# PK-6100) in 0.1M PB for 1h. Sections were rinsed, and incubated with Alexa Fluor 488 streptavidin (1:400; Life Technologies, Cat# S-32354) in BS for 1h.

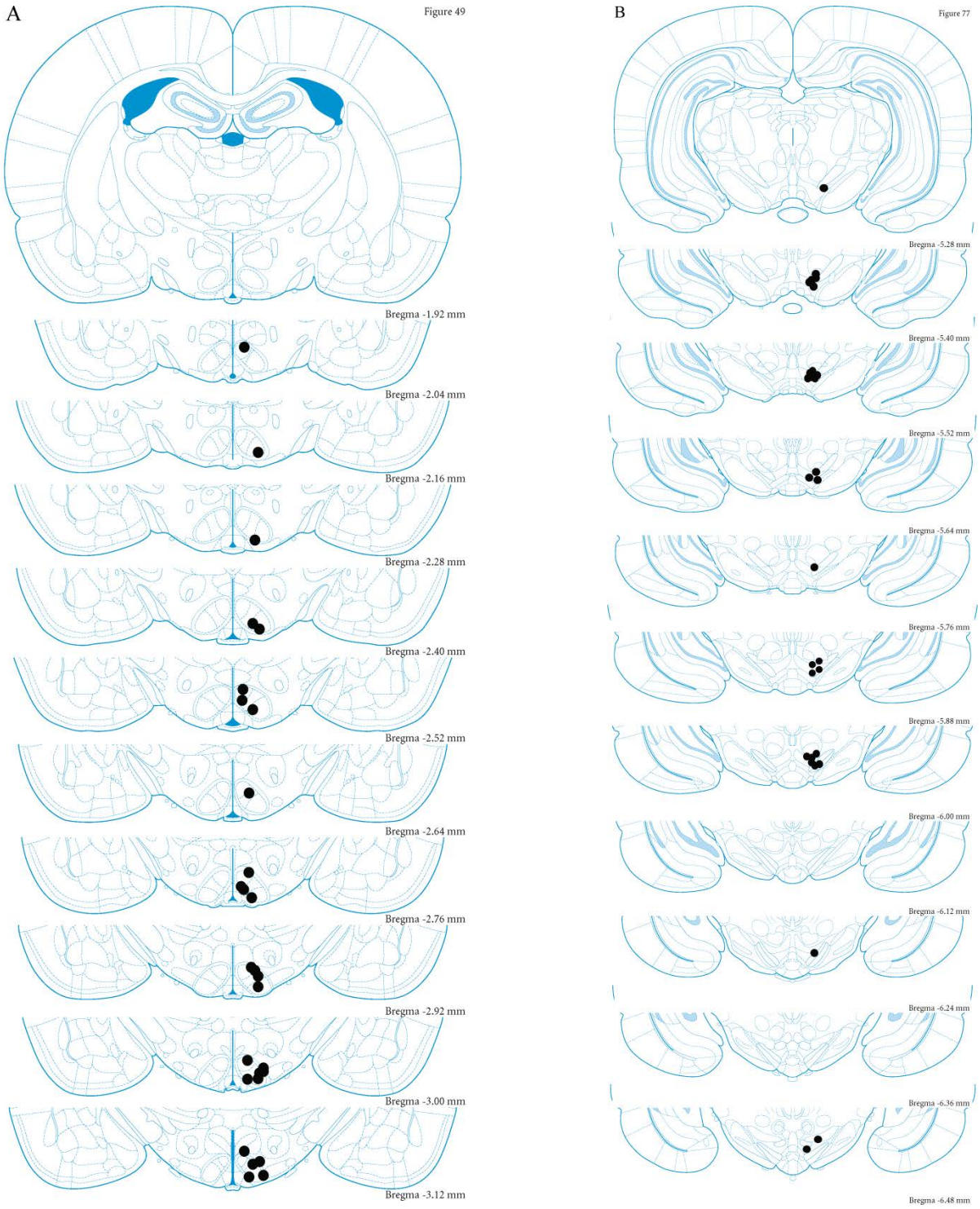

Figure 1. Cannula placements according to the atlas of Paxinos and Watson (2005) for Fluoro-Gold infusions into the (A) VMH and (B) VTA.

The neurotransmitter GABA was visualized in the same way as GLU, with the added step of incubating the sections with biotin tyramide (1:1000; Life Technologies, Cat# T20947) and 3% hydrogen peroxide (1:1000) in 0.1M PB for 10min following incubation with the ABC-elite kit. Sections were then rinsed once for 1min, followed by 5 more rinses of 5min each, before continuing on with the Alexa Fluor 488 streptavidin incubation.

The procedure for visualizing DAR continued immediately following the GLU or GABA FIHC. Following another rinse, sections were incubated overnight in BS with a primary antibody raised in rabbit recognizing either DA D1R (1:800; Calbiochem, Cat# 324390) or DA D2R (1:800; Calbiochem, Cat# 324393). The next day, sections were rinsed, and incubated with Alexa Fluor 594-tagged goat anti-rabbit IgG (1:200; Life Technologies, Cat# A-11012) in BS for 1h. Sections were rinsed, mounted on gel-coated slides, and allowed to dry overnight at room temperature. The following day sections were coverslipped with Fluoromount (Sigma-Aldrich, Cat# F4680), and stored at 4ºC until microscopy. Control sections for all antibodies included omission of the primary antibody, which resulted in no staining.

Imaging

Microscopy imaging was performed with an Olympus Fluoview FV10i confocal laser scanning microscope (Olympus, USA) fitted with 60x/1.35 oil (pixel size 0.207µm). A 405nm laser combined with a Blue filter (Em 470nm), a 473nm laser with Alexa488 filter (Em 520nm) and a 559nm laser with Alexa594 filter (Em 618nm) were used to image the proteins associated with FG, the neurotransmitter of interest (GABA or GLU), and the DAR of interest (D1R or D2R), respectively. The 0.75µm spacing z-stacks were converted to sum z-projections using Fiji software (Schindelin, Arganda-Carreras, Frise, Kaynig, Longair, Pietzsch, Preibisch, Rueden, Saalfeld, Schmid, Tinevez, White, Hartenstein, Eliceiri, Tomancak, & Cardona, 2012). 3D reconstruction and objects counting (Spots tool) were created from 25µm Z-stacks with the software IMARIS 8.1 (Bitplane, Switzerland (http://www.bitplane.com)). Representative images of the different fluorescent staining can be seen in Figure 2.

Cell counting was performed by taking 6 randomized counts per animal and creating an average count. These averaged counts were then combined for animals in each hormonal group, creating adjusted densities. Images used to count were randomized throughout the mPOA, but effort was made to find the largest amount of FG-positive neurons, as these were the least frequent.

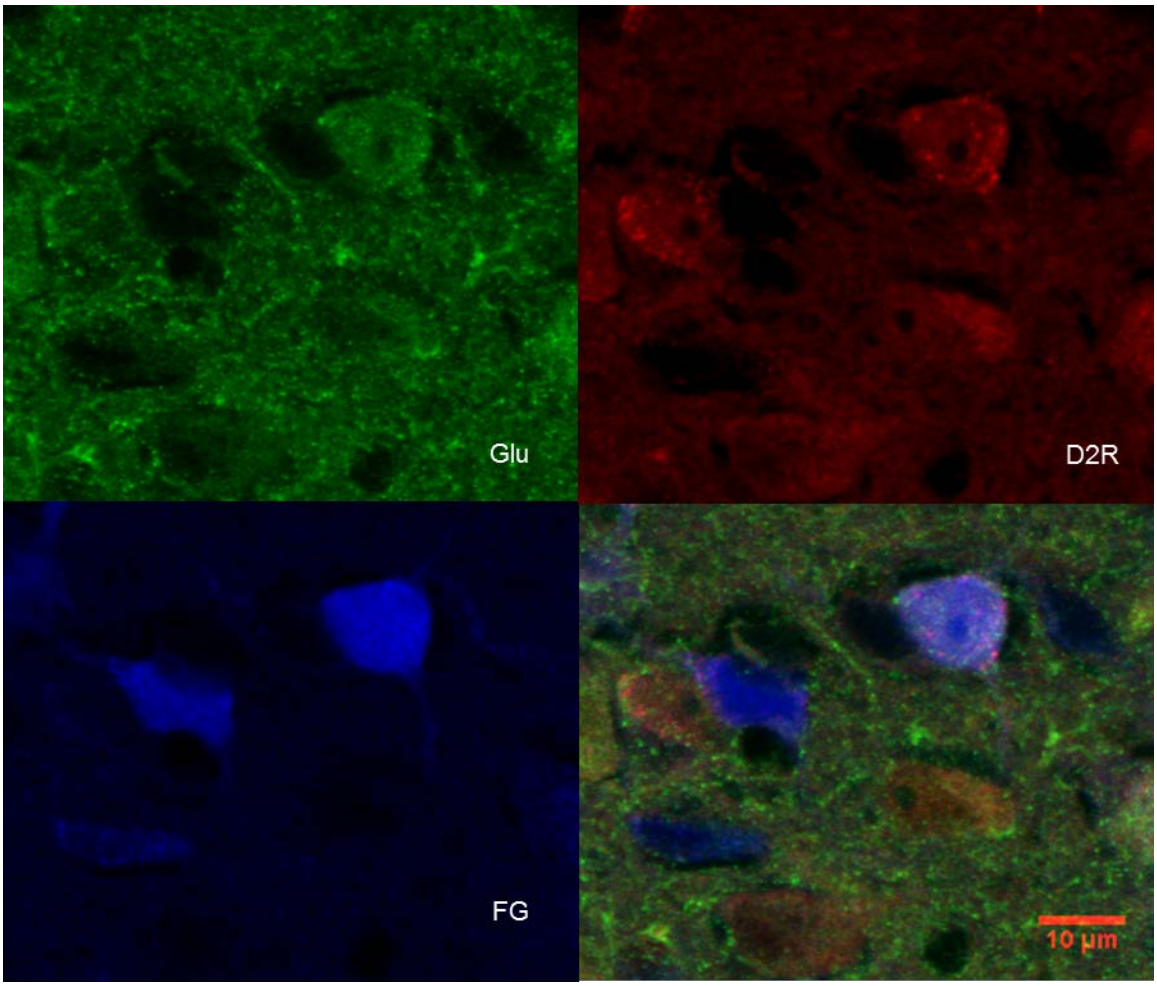

Figure 2. Representative fluorescent immunohistochemistry images, including an example of a triple-labelled cell.

Statistical Analyses

To compare the three hormonal groups (O+O, EB+O, EB+P) on the log-transformed D1R/D2R ratio and adjusted densities a 3 X 3 mixed-design ANOVA was used. The within-subject factor consisted of brain slice sample (three slices taken at random); the between-groups factor was hormonal group (O+O, EB+O, EB+P). A one-way ANOVA was used to compare the proportions of neurons double- and triple-labelled for the different fluorescent-stained proteins across the three hormonal groups, after the proportions were averaged across samples. For all ANOVAs, groups were combined for specific analyses whenever possible to increase power, and any significant omnibus F-test was further analyzed using a Least Significant Difference post hoc analysis to determine which of the hormonal groups differed. Analyses of D1R/D2R ratio effects were performed using log transformations, so that the data would adhere to the assumption of linearity. Effect sizes for the log-transformed D1R/D2R ratio and adjusted densities were calculated using partial eta squared due to the mixed-design ANOVA employed, while eta squared effect sizes were calculated for the proportion analyses. For all analyses, a 90% confidence interval was calculated around the effect size (Steiger, 2004). Due to the paucity of previous effect size reports, all effect size interpretations were compared only within the confines of the present studies (for detailed explanation, see Thompson, 2007). Thus, .100, .200, and .300 were used as the criteria for small, medium, and large effect sizes, respectively.

Results

DAR Ratio

A statistically significant effect of hormonal group on the log-transformed D1R/D2R ratio was found ($F(2, 57) = 3.817$, $p = .028$, $\eta_p^2 = .118$, 90% CI [.007, .236]), with a small to medium effect size. Post hoc analyses revealed that the EB+O group had a significant decrease in log-transformed D1R/D2R ratio compared to the other two groups, indicating that the EB+O group had a DAR ratio more tilted towards D2R (Figure 3).

Adjusted Densities

Overall, no significant differences were found for the adjusted densities of any of the labelling techniques (single, double, or triple). This was most likely due to the time constraint of the imaging procedure where we examined only a small section of each random slice, and thus

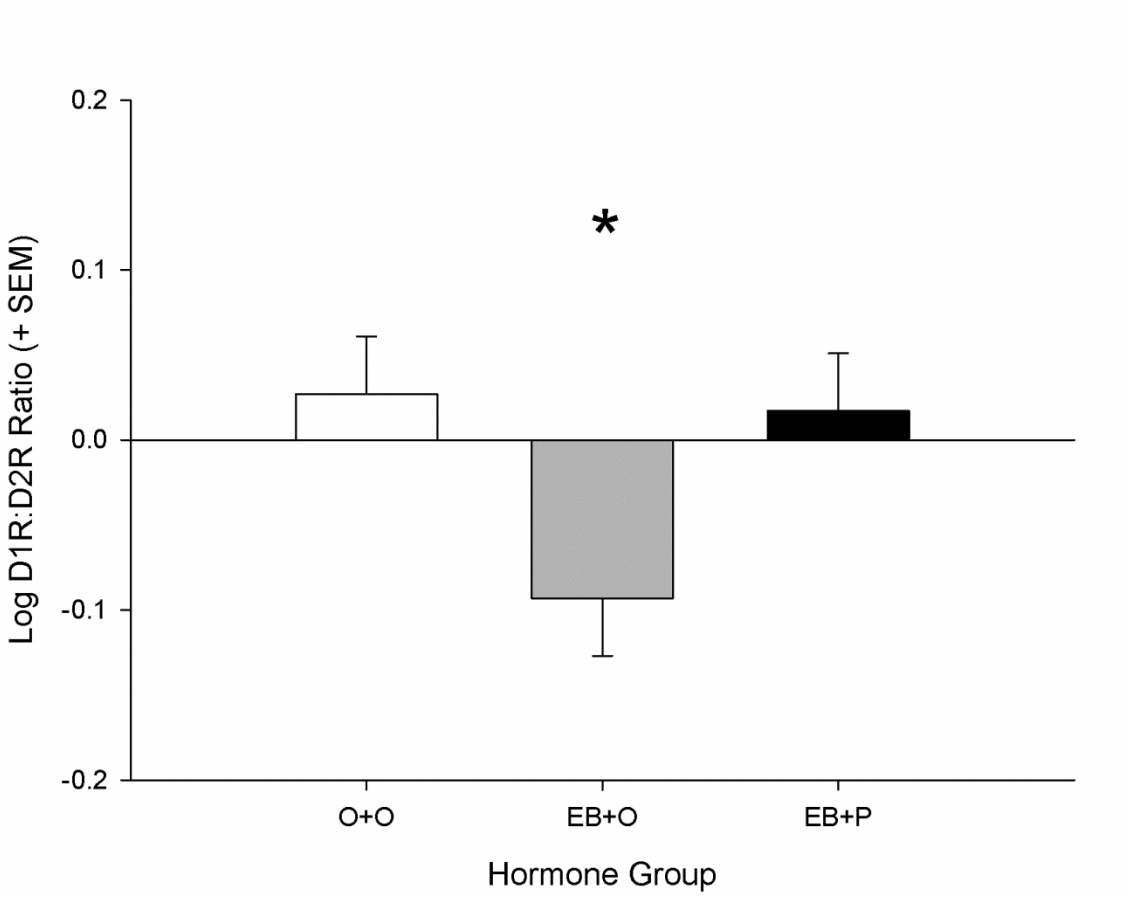

Figure 3. Analysis of hormonal group on the log-transformed D1R/D2R standardized count ratio. Each value represents the mean standardized count ratio, with error bars representing the SEM. Females primed with EB + O had a significant decrease in log-transformed D1R/D2R ratio compared with females of the other two groups. * $p < .05$.

each section represents only a portion of the entire mPOA. Raw numbers for all adjusted densities are shown in Table 1.

Single-Labelling

No significant effect of hormonal group was found on overall FG staining projecting to the VMH ($F(2, 27) = 2.158$, $p = .135$, $\eta_p^2 = .138$, 90% CI [.000, .300]) or the VTA ($F(2, 27) = 0.244$, $p = .785$, $\eta_p^2 = .018$, 90% CI [.000, .100]), with effect sizes ranging from very small to small-medium. Similarly, there was no significant effect of hormonal group on GABA staining ($F(2, 27) = 0.004$, $p = .996$, $\eta_p^2 = .000$, 90% CI [.000, .006]) or GLU staining ($F(2, 27) = 0.450$, $p = .642$, $\eta_p^2 = .032$, 90% CI [.000, .142]), with both neuronal stainings showing very small effect sizes. Finally there was also no significant effect of hormonal group on D1R staining ($F(2, 57) = 1.161$, $p = .320$, $\eta_p^2 = .039$, 90% CI [.000, .126]) or D2R staining ($F(2, 57) = 0.184$, $p = .832$, $\eta_p^2 = .006$, 90% CI [.000, .042]), with both DAR showing very small effect sizes.

Double-Labelling

FG & Neurotransmitter. There was a trend towards a significant difference in hormonal groups on the adjusted number of neurons projecting to the VMH that were double-labelled with GABA ($F(2, 12) = 2.984$, $p = .089$, $\eta_p^2 = .332$, 90% CI [.000, .526]), and with GLU ($F(2, 12) = 2.879$, $p = .095$, $\eta_p^2 = .324$, 90% CI [.000, .520]), with large effect sizes being observed in both circumstances (See Table 1). Specifically, rats receiving EB+O had a higher adjusted density of GABA labelled-neurons projecting to the VMH than the other two groups (Figure 4A). Additionally, rats receiving only oil had a higher adjusted density of GLU labelled-neurons projecting to the same area, indicating that the administration of EB with or without P decreases this density (Figure 4B). No significant differences were found for FG neurons projecting to the VTA double-labelled with GABA ($F(2, 12) = 0.801$, $p = .471$, $\eta_p^2 = .118$, 90% CI [.000, .320]) or GLU ($F(2, 12) = 0.413$, $p = .671$, $\eta_p^2 = .064$, 90% CI [.000, .240]), with small to medium effect sizes observed.

FG & DAR. No significant differences by hormonal group were found for double-labelled neurons projecting to the VMH that also were stained for D1R ($F(2, 27) = 1.882$, $p = .172$, $\eta_p^2 = .122$, 90% CI [.000, .282]) or D2R ($F(2, 27) = 1.678$, $p = .206$, $\eta_p^2 = .111$, 90% CI

Table 1. Adjusted density raw numbers organized by hormonal group.

	ADJUSTED DENSITIES				
	O+O	EB+O	EB+P	*p*	partial η^2
Single-Labelling					
VMH FG	23.037	7.300	6.630	.135	.138
VTA FG	19.099	23.540	24.727	.785	.018
GABA	127.568	125.918	127.588	.996	.000
GLU	155.867	137.254	162.840	.642	.032
D1R	220.932	188.085	208.216	.320	.039
D2R	212.711	222.404	207.204	.832	.006
Double-Labelling					
VMH FG & GABA	0.215	0.983	0.185	.089 #	.332 ^
VMH FG & GLU	2.693	0.346	0.614	.095 #	.324 ^
VMH FG & D1R	9.227	1.344	1.481	.172	.122
VMH FG & D2R	4.281	2.650	1.329	.206	.111
VTA FG & GABA	0.222	0.667	0.624	.471	.118
VTA FG & GLU	0.186	0.147	0.352	.671	.064
VTA FG & D1R	8.199	12.187	7.442	.619	.035
VTA FG & D2R	15.142	13.593	17.946	.818	.015
GABA & D1R	31.274	27.770	36.402	.296	.086
GABA & D2R	29.881	30.715	31.027	.989	.001
GLU & D1R	56.324	60.063	72.468	.518	.048
GLU & D2R	51.777	49.576	62.955	.622	.035
Triple-Labelling					
VMH FG/GABA/D1R	0.804	1.273	0.743	.793	.038
VMH FG/GABA/D2R	0.155	1.496	0.779	.311	.177
VMH FG/GLU/D1R	8.875	1.634	3.531	.199	.236
VMH FG/GLU/D2R	4.568	2.867	5.659	.485	.114
VTA FG/GABA/D1R	1.006	0.834	0.402	.684	.061
VTA FG/GABA/D2R	3.472	7.486	8.357	.358	.157
VTA FG/GLU/D1R	8.078	5.992	6.414	.831	.030
VTA FG/GLU/D2R	1.969	2.166	5.125	.478	.116

Note: # $p < .10$, ^ $\eta^2 > .300$.

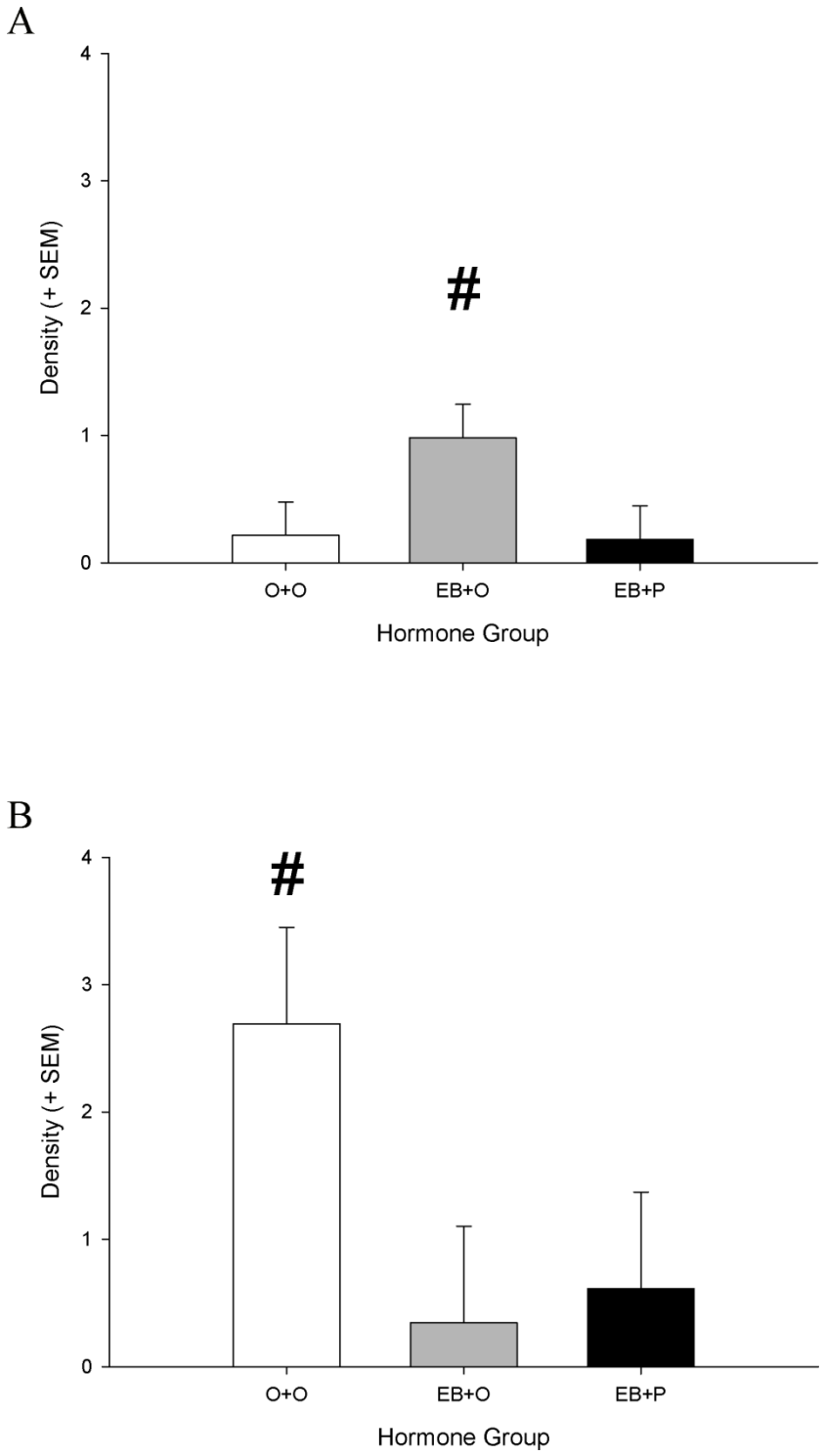

Figure 4. Analysis of hormonal group on adjusted density of FG-stained cells projecting to the VMH double-labelled with either (A) GABA or (B) glutamate. Each value represents the mean adjusted density level. Error bars represent the SEM. # $p < .10$.

[.000, .267]), with slightly above small effect sizes observed. This was also found for neurons projecting to the VTA double-labelled with D1R ($F(2, 27) = 0.488$, $p = .619$, $\eta_p^2 = .035$, 90% CI [.000, .148]) or D2R ($F(2, 27) = 0.202$, $p = .818$, $\eta_p^2 = .015$, 90% CI [.000, .099]), though in contrast very small effect sizes were observed.

Neurotransmitter & DAR. No significant differences by hormonal group were found for any of the combinations of neurons stained for the neurotransmitter of interest double-labelled with either DAR staining. Specifically no differences were found for GABA staining double-labelled with either D1R ($F(2, 27) = 1.272$, $p = .296$, $\eta_p^2 = .086$, 90% CI [.000, .235]) or D2R ($F(2, 27) = 0.011$, $p = .989$, $\eta_p^2 = .001$, 90% CI [.000, .016]), nor for GLU staining doubled-labelled with either D1R ($F(2, 27) = 0.675$, $p = .518$, $\eta_p^2 = .048$, 90% CI [.000, .174]) or D2R ($F(2, 27) = 0.483$, $p = .622$, $\eta_p^2 = .035$, 90% CI [.000, .147]), with very small effect sizes found in every double-labelled combination.

Triple-Labelling

None of the eight combinations of triple-labelling fluorescent stainings were found to be significantly different between the three hormonal groups, although varied effect sizes were observed. When examining neurons projecting to the VMH, triple-labelling involving GABA and D1R had a very small effect size ($F(2, 12) = 0.237$, $p = .793$, $\eta_p^2 = .038$, 90% CI [.000, .193]), but those triple-labelled with GLU and D1R had a much stronger effect size, in the medium range ($F(2, 12) = 1.854$, $p = .199$, $\eta_p^2 = .236$, 90% CI [.000, .445]). As far as neurons stained with FG projecting to the VMH involving D2R, there were similar small to medium effect sizes observed when triple-labelled with either GABA ($F(2, 12) = 1.291$, $p = .311$, $\eta_p^2 = .177$, 90% CI [.000, .388]) or GLU ($F(2, 12) = 0.768$, $p = .485$, $\eta_p^2 = .114$, 90% CI [.000, .315]). In regards to neurons projecting to the VTA, those triple-labelled with D1R had a very small effect size when also staining for GABA ($F(2, 12) = 0.392$, $p = .684$, $\eta_p^2 = .061$, 90% CI [.000, .234]) and GLU ($F(2, 12) = 0.188$, $p = .831$, $\eta_p^2 = .030$, 90% CI [.000, .175]). A small to medium effect size was seen when triple-labelling with D2R when also staining for GABA ($F(2, 12) = 1.120$, $p = .358$, $\eta_p^2 = .157$, 90% CI [.000, .367]) or GLU ($F(2, 12) = 0.786$, $p = .478$, $\eta_p^2 = .116$, 90% CI [.000, .318]).

Proportions

Proportions of stained proteins double- and triple-labelled were calculated and compared across hormonal groups. Calculated proportions are depicted in Table 2.

Proportion of Double-Labelled Cells Projecting to the VMH. Examination of double-labelling proportions of cells projecting to the VMH for the three hormonal groups indicate trends towards significance with large effect sizes. This was found for the proportion of VMH FG cells double-labelled with GABA ($F(2, 12) = 3.329$, $p = .071$, $\eta^2 = .357$, 90% CI [.000, .546]), with both groups that received EB (with or without P) having a higher proportion than the oil group (Figure 5A). This was also the case for the proportion of VMH FG cells double-labelled with GLU ($F(2, 12) = 2.971$, $p = .090$, $\eta^2 = .331$, 90% CI [.000, .525]), with rats receiving EB+P having a trend towards being significantly higher than the oil control group (Figure 5B). There was also a trend towards significance for the proportion of GABA-labelled cells double-labelled with the presence of FG, indicating they also project to the VMH ($F(2, 12) = 2.816$, $p = .099$, $\eta^2 = .319$, 90% CI [.000, .516]), with a large effect size found. Here, the EB+O group was trending towards being significantly higher than the O+O group (Figure 6). In each of these above trends effect sizes were found to be large, with partial η^2 scores of at least .319. No significant difference was found for hormonal group on the proportion of GLU-labelled cells double-labelled with FG ($F(2, 12) = 1.292$, $p = .310$, $\eta^2 = .177$, 90% CI [.000, .388]), with a small to medium effect size observed.

No significant differences were found by hormonal group for the proportion of VMH/FG stained cells double-labelled with either D1R ($F(2, 23) = 0.629$, $p = .542$, $\eta^2 = .052$, 90% CI [.000, .189]) nor D2R ($F(2, 26) = 1.935$, $p = .165$, $\eta^2 = .130$, 90% CI [.000, .293]), with small effect sizes found for both. Similarly, no significant differences were found for the proportion of D1R-stained cells double-labelled with FG ($F(2, 27) = 1.778$, $p = .188$, $\eta^2 = .116$, 90% CI [.000, .274]), nor for the proportion of D2R-stained cells double-labelled with FG ($F(2, 27) = 0.538$, $p = .590$, $\eta^2 = .038$, 90% CI [.000, .155]), with small effect sizes found.

Table 2: Raw proportions organized by hormonal group.

	PROPORTIONS				
	O+O	EB+O	EB+P	*p*	η^2
VMH Double-Labelling					
FG DL with GABA	17.1%	43.6%	48.2%	.071 #	.357 ^
FG DL with GLU	30.9%	41.3%	62.6%	.090 #	.331 ^
GABA DL with FG	0.8%	2.6%	1.3%	.099 #	.319 ^
GLU DL with FG	9.9%	3.7%	4.4%	.310	.177
FG DL with D1R	51.1%	45.6%	62.4%	.542	.052
FG DL with D2R	37.7%	46.3%	61.4%	.165	.165
VTA Double-Labelling					
FG DL with GABA	19.5%	12.3%	13.6%	.310	.177
FG DL with GLU	45.6%	32.7%	42.5%	.662	.066
GABA DL with FG	1.4%	2.9%	2.7%	.394	.144
GLU DL with FG	2.8%	2.2%	3.7%	.710	.055
FG DL with D1R	95.8%	88.2%	91.9%	.292	.116
FG DL with D2R	81.1%	75.7%	72.4%	.540	.048
Combined Double-Labelling					
GABA DL with D1R	25.1%	24.3%	29.4%	.462	.056
GABA DL with D2R	26.4%	29.6%	31.4%	.652	.031
GLU DL with D1R	37.7%	40.9%	43.2%	.640	.033
GLU DL with D2R	40.1%	45.6%	48.8%	.364	.072
D1R DL with GABA	16.9%	22.1%	23.2%	.379	.069
D1R DL with GLU	26.5%	26.3%	32.4%	.328	.079
D2R DL with GABA	14.6%	15.7%	16.7%	.788	.018
D2R DL with GLU	24.9%	22.8%	34.5%	.051 #	.198
VMH Triple-Labelling					
FG TL with GABA & D1R	25.0%	21.4%	46.9%	.388	.190
FG TL with GABA & D2R	7.1%	18.3%	36.7%	.163	.281
FG TL with GLU & D1R	21.5%	44.2%	44.1%	.353	.173
FG TL with GLU & D2R	24.1%	27.6%	58.0%	.042 *	.412 ^
GABA TL with FG & D1R	0.7%	1.1%	0.8%	.891	.019
GABA TL with FG & D2R	0.2%	1.9%	1.4%	.331	.168
GLU TL with FG & D1R	7.0%	2.0%	2.7%	.231	.217
GLU TL with FG & D2R	5.1%	4.6%	4.9%	.986	.002
D1R TL with FG & GABA	0.6%	1.3%	0.7%	.620	.077
D1R TL with FG & GLU	3.8%	0.8%	1.8%	.184	.246
D2R TL with FG & GABA	0.1%	0.7%	0.8%	.315	.175
D2R TL with FG & GLU	2.9%	1.7%	3.1%	.562	.092

	O+O	EB+O	EB+P	*p*	η^2
VTA Triple-Labelling					
FG TL with GABA & D1R	26.0%	2.8%	5.0%	.106	.527 ^^
FG TL with GABA & D2R	13.1%	13.1%	14.8%	.922	.013
FG TL with GLU & D1R	36.6%	28.3%	36.0%	.626	.082
FG TL with GLU & D2R	42.5%	42.1%	46.6%	.977	.005
GABA TL with FG & D1R	0.6%	0.5%	0.2%	.666	.066
GABA TL with FG & D2R	2.1%	4.5%	4.3%	.277	.193
GLU TL with FG & D1R	3.7%	2.9%	2.9%	.808	.035
GLU TL with FG & D2R	1.6%	1.4%	4.0%	.496	.110
D1R TL with FG & GABA	0.4%	0.4%	0.2%	.739	.049
D1R TL with FG & GLU	3.3%	2.4%	2.5%	.776	.041
D2R TL with FG & GABA	1.2%	2.6%	2.8%	.307	.179
D2R TL with FG & GLU	1.1%	0.9%	2.7%	.484	.114

Note: * $p < .05$, # $p < .10$, ^ $\eta^2 > .300$, ^^ $\eta^2 > .500$.

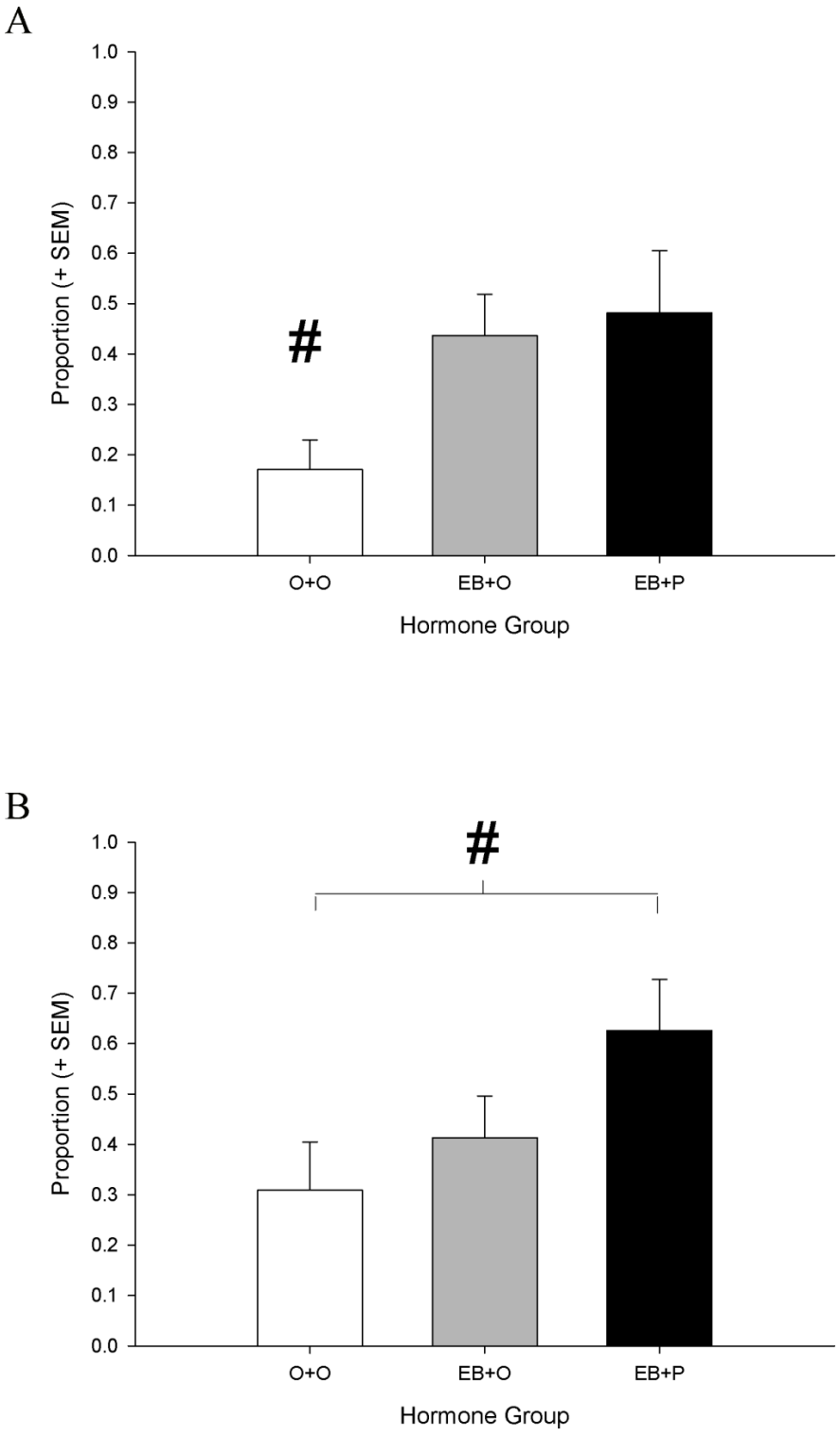

Figure 5. Analysis of hormonal group on the proportion of FG-stained cells projecting to the VMH double-labelled with either (A) GABA or (B) glutamate. Each value represents the mean proportion level. Error bars represent the SEM. # $p < .10$.

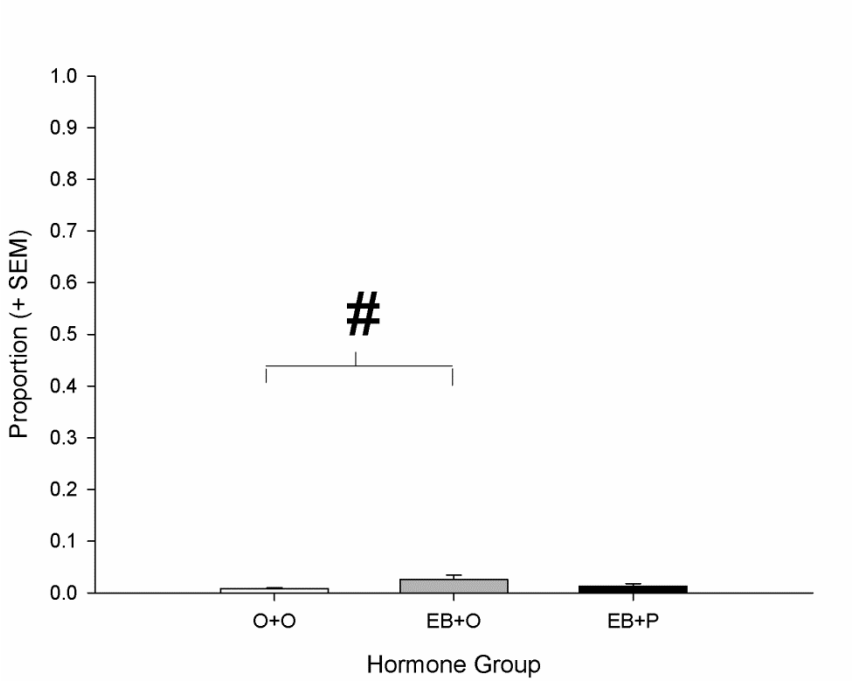

Figure 6. Analysis of hormonal group on the proportion of GABA-stained cells projecting to the VMH. Each value represents the mean proportion level. Error bars represent the SEM. # $p <$ .10.

Proportion of Double-Labelled Cells Projecting to the VTA. Examination of double-labelling proportions with cells projecting to the VTA revealed no significant differences for the three hormonal groups. This includes the proportions of cells stained for VTA FG double-labelled with GABA ($F(2, 12) = 1.292$, $p = .310$, $\eta^2 = .177$, 90% CI [.000, .388]), GLU ($F(2, 12) = 0.426$, $p = .662$, $\eta^2 = .066$, 90% CI [.000, .243]), D1R ($F(2, 20) = 1.309$, $p = .292$, $\eta^2 = .116$, 90% CI [.000, .291]), and D2R ($F(2, 25) = 0.632$, $p = .540$, $\eta^2 = .048$, 90% CI [.000, .178]), with effect sizes in the small to medium range. Likewise, neither of the proportions of GABA-stained cells double-labelled with VTA FG ($F(2, 12) = 1.008$, $p = .394$, $\eta^2 = .144$, 90% CI [.000, .352]) nor GLU-stained cells double-labelled with VTA FG ($F(2, 12) = 0.352$, $p = .710$, $\eta^2 = .055$, 90% CI [.000, .222]) were significantly different, with effect sizes again being small. Finally, neither of the proportions of D1R-stained cells double-labelled with VTA FG ($F(2, 27) = 0.270$, $p = .766$, $\eta^2 = .020$, 90% CI [.000, .106]) nor D2R-stained cells double-labelled with VTA FG ($F(2, 27) = 0.306$, $p = .739$, $\eta^2 = .022$, 90% CI [.000, .114]) were significantly different, with very small effect sizes observed.

Proportion of Double-Labelled Cells Overall. Double-labelling proportions were examined between cells showing neurotransmitter staining (GABA or GLU) with the two DARs (D1R or D2R), combined across studies. A trend towards significance was found for the proportion of cells stained for D2R double-labelled with GLU-staining ($F(2, 27) = 3.334$, $p = .051$, $\eta^2 = .198$, 90% CI [.000, .365]), with a medium effect size found. Rats receiving EB+P had a higher proportion of these double-labelled cells than the other two groups (Figure 7). No other significant differences were discovered. This includes the proportion of GABA-stained cells double-labelled with D1R ($F(2, 27) = 0.794$, $p = .462$, $\eta^2 = .056$, 90% CI [.000, .188]) or D2R ($F(2, 27) = 0.435$, $p = .652$, $\eta^2 = .031$, 90% CI [.000, .139]), the proportion of GLU-stained cells double-labelled with D1R ($F(2, 27) = 0.454$, $p = .640$, $\eta^2 = .033$, 90% CI [.000, .142]) or D2R ($F(2, 27) = 1.048$, $p = .364$, $\eta^2 = .072$, 90% CI [.000, .214]), the proportion of D1R-stained cells double-labelled with GABA ($F(2, 27) = 1.007$, $p = .379$, $\eta^2 = .069$, 90% CI [.000, .210]) or GLU ($F(2, 27) = 1.163$, $p = .328$, $\eta^2 = .079$, 90% CI [.000, .225]), and finally D2R-stained cells double-labelled with GABA ($F(2, 27) = 0.241$, $p = .788$, $\eta^2 = .018$, 90% CI [.000, .099]). Each effect size for these non-significant differences ranged from very small to small.

Proportion of Triple-Labelled Cells in the VMH. Examination of the proportion of cells that showed all three types of staining were compared between the three hormonal groups. There

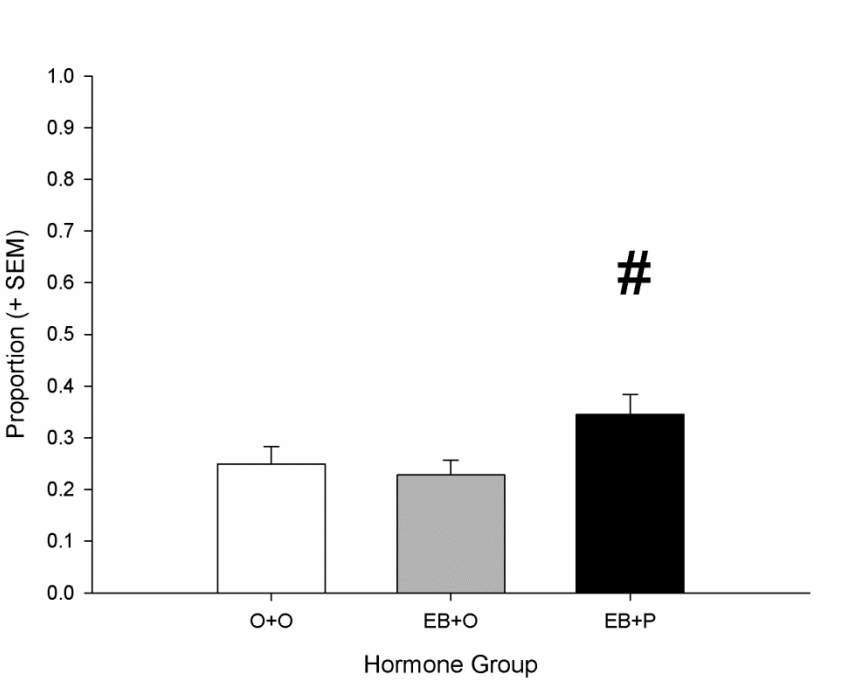

Figure 7. Analysis of hormonal group on the proportion of D2R-stained cells doubled-labelled with GLU-staining. Each value represents the mean proportion level. Error bars represent the SEM. # $p < .10$.

was a significant difference found for the proportion of cells projecting to the VMH that also showed staining with GLU and D2R ($F(2, 12) = 4.196$, $p = .042$, $\eta^2 = .412$, 90% CI [.011, .588], with a very large effect size (Figure 8). Post hoc comparisons found that rats primed with both EB+P showed a greater proportion than the other two groups. The proportion of cells projecting to the VMH triple-labelled with GLU and D1R was not significant ($F(2, 11) = 1.147$, $p = .353$, $\eta^2 = .173$, 90% CI [.000, .387]), nor were the proportions of VMH projecting cells triple-labelled with GABA and D1R ($F(2, 9) = 1.054$, $p = .388$, $\eta^2 = .190$, 90% CI [.000, .413]) or GABA and D2R ($F(2, 11) = 2.149$, $p = .163$, $\eta^2 = .281$, 90% CI [.000, .488]), though in all three cases the effect sizes were medium to large in size, indicating that perhaps power was too low to find a statistical difference.

The proportion of GABA-stained cells triple-labelled with cells projecting to the VMH and D1R was not significantly different ($F(2, 12) = 0.116$, $p = .891$, $\eta^2 = .019$, 90% CI [.000, .143]) and the effect size was very small. A bigger effect size was found, in the medium range, when comparing the proportion of GABA-stained cells triple-labelled with cells projecting to the VMH and D2R, though again this was not statistically significantly different ($F(2, 12) = 1.214$, $p = .331$, $\eta^2 = .168$, 90% CI [.000, .379]). Similar non-significant results were found when examining the proportion of GLU-stained triple-labelled with cells projecting to the VMH and D1R ($F(2, 12) = 1.662$, $p = .231$, $\eta^2 = .217$, 90% CI [.000, .427]) and for the proportion of GLU-stained triple-labelled with cells projecting to the VMH and D2R ($F(2, 12) = 0.014$, $p = .986$, $\eta^2 = .002$, 90% CI [.000, .039]), though the effect sizes were in the opposite pattern for DAR.

The proportion of D1R-stained cells triple-labelled with cells projecting to the VMH and GABA was not significantly different between the hormone groups ($F(2, 12) = 0.497$, $p = .620$, $\eta^2 = .077$, 90% CI [.000, .261]), with a small effect sizes. Although the proportion of D1R-stained cells triple-labelled with cells projecting to the VMH and GLU was also not significantly different between hormone groups ($F(2, 12) = 1.957$, $p = .184$, $\eta^2 = .246$, 90% CI [.000, .453]) the effect size was in the medium to large range, again indicating that perhaps low power was an issue. Analysis of the proportion of D2R-stained cells triple-labelled also indicated no significant differences between hormone groups when triple-labelled with VMH FG and GABA ($F(2, 12) = 1.275$, $p = .315$, $\eta^2 = .175$, 90% CI [.000, .386]) nor when triple-labelled with VMH FG and GLU ($F(2, 12) = 0.604$, $p = .562$, $\eta^2 = .092$, 90% CI [.000, .285]), with effect sizes in the medium and small range, respectively.

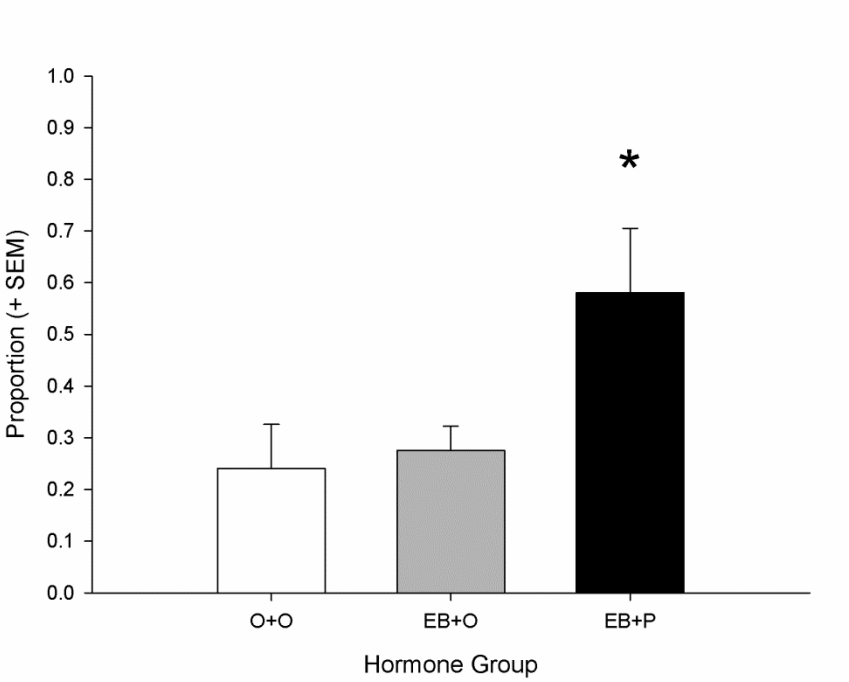

Figure 8. Analysis of hormonal group on the proportion of FG-stained cells projecting to the VMH triple-labelled for glutamate and D2R. Each value represents the mean proportion level. Error bars represent the SEM. * $p < .05$.

Proportion of Triple-Labelled Cells in the VTA. Analysis of the proportion of cells projecting to the VTA triple-labelled with GABA and D1R showed a trend towards significance but a very large effect size ($F(2, 6) = 3.344$, $p = .106$, $\eta^2 = .527$, 90% CI [.000, .683]), once again indicating that low power was a potential problem for the statistical analyses of the proportion data. Nevertheless rats in the oil control group were much higher than the hormonal manipulation groups (Figure 9). All other analyses examining triple-labelled proportions of VTA-projecting cells showed very small effect sizes and non-significant differences, specifically the proportion triple-labelled with GABA and D2R ($F(2, 12) = 0.082$, $p = .922$, $\eta^2 = .013$, 90% CI [.000, .124]), GLU and D1R ($F(2, 11) = 0.489$, $p = .626$, $\eta^2 = .082$, 90% CI [.000, .273]), and GLU and D2R ($F(2, 10) = 0.023$, $p = .977$, $\eta^2 = .005$, 90% CI [.000, .071]).

The proportion of GABA-stained cells triple-labelled with VTA FG and D1R was not significantly different between hormone groups ($F(2, 12) = 0.421$, $p = .666$, $\eta^2 = .066$, 90% CI [.000, .242]), with a small effect size, as was the proportion triple-labelled with VTA FG and D2R ($F(2, 12) = 1.431$, $p = .277$, $\eta^2 = .193$, 90% CI [.000, .403]), though a medium effect size was found there. Similarly, non-significant differences and small effect sizes were found for the proportion of GLU-stained cells tripled-labelled with VTA FG and D1R ($F(2, 12) = 0.217$, $p = .808$, $\eta^2 = .035$, 90% CI [.000, .186]) and with VTA FG and D2R ($F(2, 12) = 0.744$, $p = .496$, $\eta^2 = .110$, 90% CI [.000, .311]).

Finally non-significant differences and small effect sizes were found for the proportion of D1R-stained cells triple-labelled with VTA FG and GABA ($F(2, 12) = 0.310$, $p = .739$, $\eta^2 = .049$, 90% CI [.000, .208]) and with VTA FG and GLU ($F(2, 12) = 0.259$, $p = .776$, $\eta^2 = .041$, 90% CI [.000, .200]). Similar non-significant findings were found for the proportion of D2R-stained cells, though the effect sizes were slightly larger, though still in the small to medium range, for cells triple-labelled with VTA FG and GABA ($F(2, 12) = 1.304$, $p = .307$, $\eta^2 = .179$, 90% CI [.000, .389]) and VTA FG and GLU ($F(2, 12) = 0.772$, $p = .484$, $\eta^2 = .114$, 90% CI [.000, .316]).

Discussion

This experiment examined neural connections projecting from the medial preoptic area to two areas previously implicated in the control of female sexual behaviour, the VMH and VTA, under different hormonal priming conditions. The aim was to determine what proportion were

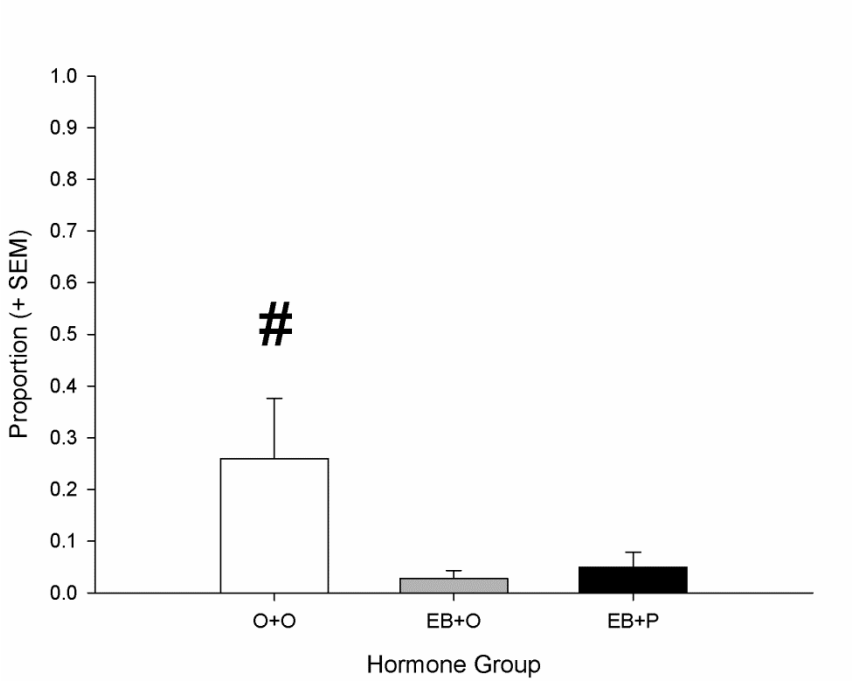

Figure 9. Analysis of hormonal group on the proportion of FG-stained cells projecting to the VTA triple-labelled for GABA and D1R. Each value represents the mean proportion level. Error bars represent the SEM. # $p < .10$.

glutamatergic or GABAergic, and whether dopamine receptors were present on their cell bodies in the mPOA. This was done given our previous data showing hormonal changes in D1R/D2R ratio in the mPOA controls the display of appetitive and receptive sexual behaviours in female rats (Graham et al., 2015), consistent with pharmacological effects on the two receptor subtypes in the mPOA (Graham & Pfaus, 2010; Graham & Pfaus, 2012). Using immunofluorescence staining, we replicated the hormonal effect found previously for D1R/D2R ratios, as females primed with EB+O displayed a lower D1R/D2R ratio than those primed with O+O or EB+P. Specifically, in females primed with EB+O, a low D1R/D2R ratio was found, as replicated here, and animals in this state display low levels of lordosis, and minimal appetitive sexual behaviours. Additional neurotransmission, for example in the form of augmented D2R binding, increases these appetitive sexual behaviours (Graham & Pfaus, 2010). When P is given in addition to EB, the pattern switches to a higher D1R/D2R ratio level, and high amounts of appetitive sexual behaviour are observed (Graham & Pfaus, 2012; Graham et al., 2015). This change in behavioural output is likely due to the interaction of steroid hormones and pathways connecting the mPOA to areas responsible for both appetitive responses like solicitations, and consummatory responses like lordosis, the VTA and VMH, respectively (reviewed in Pfaus et al., 2015).

In addition to DARs, immunofluorescence was used to examine GLU or GABA projections from the mPOA to the VMH or VTA, using FG as the retrograde tracer. Although a lack of power appeared to be an issue in terms of observing significant differences using null hypothesis significance testing, an examination of effect sizes revealed that the neurons originating in the mPOA of females primed with EB+P projecting to the VMH are more often GLU than GABA neurons, and in addition they are more likely to possess D2R. Administering EB with or without P resulted in a higher proportion of the neurons projecting to the VMH being GABAergic, which is accompanied by an increase of D1R on them when P is administered after EB, whereas both EB+O and EB+P increased the proportion of D2R located on these neurons. Estradiol increases the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) in the mediobasal hypothalamus (Weiland, 1992), and in particular the GAD65 isoform in the mPOA and the GAD67 isoform in the dorsomedial hypothalamus (McCarthy, Kaufman, Brooks, Pfaff, & Schwartz-Giblin, 1995). Increasing GABA activity in the mediobasal hypothalamus (including the VMH) facilitates lordosis (McCarthy, Malik, & Feder, 1990), whereas application of

antisense oligonucleotide against both GAD isoforms to the VMH inhibits lordosis, albeit with different time courses (McCarthy, Masters, Rimvall, Schwartz-Giblin, & Pfaff, 1994). Thus, it appears that estradiol turns a proportion of glutamate neurons in the mPOA that project to the VMH into GABA neurons that can be activated by DA. Given the well-established inhibitory actions of GLU in the VMH on both solicitations and lordosis (Kow et al., 1985; Georgescu & Pfaus, 2006a; Georgescu & Pfaus, 2006b; Georgescu et al., 2009; Booth, Wayman, & Jackson, 2010; Georgescu, Cyr, & Pfaus, 2012; Jones, Farisello, Mayer-Heft, & Pfaus, 2015), the switching of neurochemical phenotype by estradiol may represent a disinhibitory mechanism that synchronizes DA-mediated female sexual desire and receptivity with ovulation. It could also be utilized by the mPOA to toggle solicitations and lordosis in real time (Madlafousek & Hliňák, 1977; McClintock, 1984; Pfaus et al., 1999), turning one off in favour of the other in a P-dependent, D1R/D2R (solicitation/lordosis)-driven behavioural sequence.

Examination of neurons projecting to the VTA yielded results that were more straightforward. Estradiol, with or without P, decreased the proportion of neurons projecting from the mPOA to the VTA that are GABAergic and have D1R on them. Ovarian hormone administration did not affect any other triple- or double-labelled proportions. Analysis of effect sizes indicate medium effects for adjusted densities of triple-labelled cells with D2R, though the proportions were very similar. The decrease in GABA input from the mPOA to the VTA could facilitate both sexual desire and receptivity by removing inhibitory tone on mesolimbic DA neurons, thereby increasing incentive motivation in sexual contexts (Becker, 1999; Pfaus et al., 1995; Yoest et al., 2014).

Finally, the proportion of GLU cells with D2R that are either intrinsic mPOA neurons, or that project to regions other than the VMH or VTA, were increased by EB+P treatment. This suggests that when females are fully primed, a larger number of GLU neurons projecting to other areas important for female sexual behaviour express D2R. It has been shown that the mPOA has projections to the periaqueductal gray and the nucleus paragigantocellularis (Marson & Foley, 2004), areas important in the spinal circuity involved with the expression of lordosis (e.g., Sakuma & Pfaff, 1979; Pfaff, Schwartz-Gilbin, McCarthy, & Kow, 1994; Pfaff, Phillips, & Rubin, 2004) and inhibition of genital reflexes (e.g., Marson & McKenna, 1990; Marson, List, & McKenna, 1992; Marson & McKenna, 1994). An increase in D2R-expressing GLU connections to these areas could be a signalling pathway through which the mPOA could releases the tonic

inhibition of urethrogenital reflex, resulting in vaginal vasocongestion (Giuliano, Allard, Compagnie, Alexandre, Droupy, & Bernabe, 2001). Another potential area to which these GLU neurons could be projecting is the arcuate nucleus (ArcN), as projections have been observed (Conrad & Pfaff, 1975; Conrad & Pfaff, 1976). This area has been shown to be critical in acting as a timing mechanism in conjunction with the mPOA, as estradiol binding to ERα stimulates β-endorphin neurons in the ArcN that project to the mPOA through metabotropic GLU receptor 1a. This causes mu opioid receptor activation, inhibiting sexual receptivity through their internalization for the duration of its action, which is provided by GABA-B receptor activation in the ArcN (as reviewed in Sinchak & Wagner, 2012). This D2R/GLU connection could be reciprocal to the ArcN, resulting in negative feedback augmenting opioid actions in the mPOA, or the connections could be part of the mPOA projections this ArcN system activates in regard to timing mechanisms that synchronize sexual desire and receptivity to ovulation, or that pace the behavioural expression of solicitations and lordosis.

CHAPTER 5: A MODEL

Sexual behaviour is multifaceted and complex, and operates under an integrative system that combines different brain areas that excite or inhibit various motivational, emotional, cognitive, and motor components. Ovarian steroid hormones act upon a majority of these areas to facilitate excitation and disinhibition of sexual responses, allowing animals to focus on appropriate and salient sexual incentive stimuli, thus properly timing their behavioural responses (Pfaus, 2009). The mPOA has been commonly theorized to be at the centre of brain systems underlying many different functions, including maternal behaviour (e.g., Numan, 1986; Numan, 1994), male sexual behaviour (e.g., Dominguez & Hull, 2005; Hull & Dominguez, 2007), and conditioned sexual responses, including sexually conditioned place and partner preferences (Pfaus, Ismail, & Coria-Avila, 2010). Does the mPOA play a similar role in female sexual behaviours?

Lordosis Circuit Background

Several brain regions have been implicated in the control of female sexual behaviour. Early lesion studies provided the first evidence of this regarding the lordosis reflex. Ovariectomized females that were decorticated still displayed lordosis, but only when provided exogenous E2 and P (Beach, 1942). Further studies demonstrated that lordosis was under the control of hypothalamic brain areas, most notably the VMH, as lesions to this area reduced the display of lordosis (Kennedy, 1963; Kennedy & Mitra, 1963). The work of Pfaff and colleagues elucidated a circuit for lordosis that consists of four "modules" or centres of control (see Figure 1) (Pfaff, 1980; Pfaff, Kow, Loose, & Flanagan-Cato, 2008). At the lowest point is the *spinal module*, responsible for the segmental stretch and flexion reflexes that occur in response to flank and perineal stimulation by the male as it palpates the flanks and thrusts its pelvis against the female's clitoris and perineum. Areas in this module receive specific sensory input from the flanks and pelvic organs (from vaginocervical and/or clitoral stimulation). The *lower brainstem module* receives these afferent stimuli and forwards the input to higher-level modules. The strength of projections from this module vary throughout the estrous cycle (Ghanima, Bennis, Rampin, & Rousseau, 2000; Ghanima, Bennis, & Rampin, 2002), and the amount and pattern of neural activation is dependent upon steroid hormone activation of spinal and supraspinal regions (Pfaus et al., 1996; Pfaus et al., 2015).

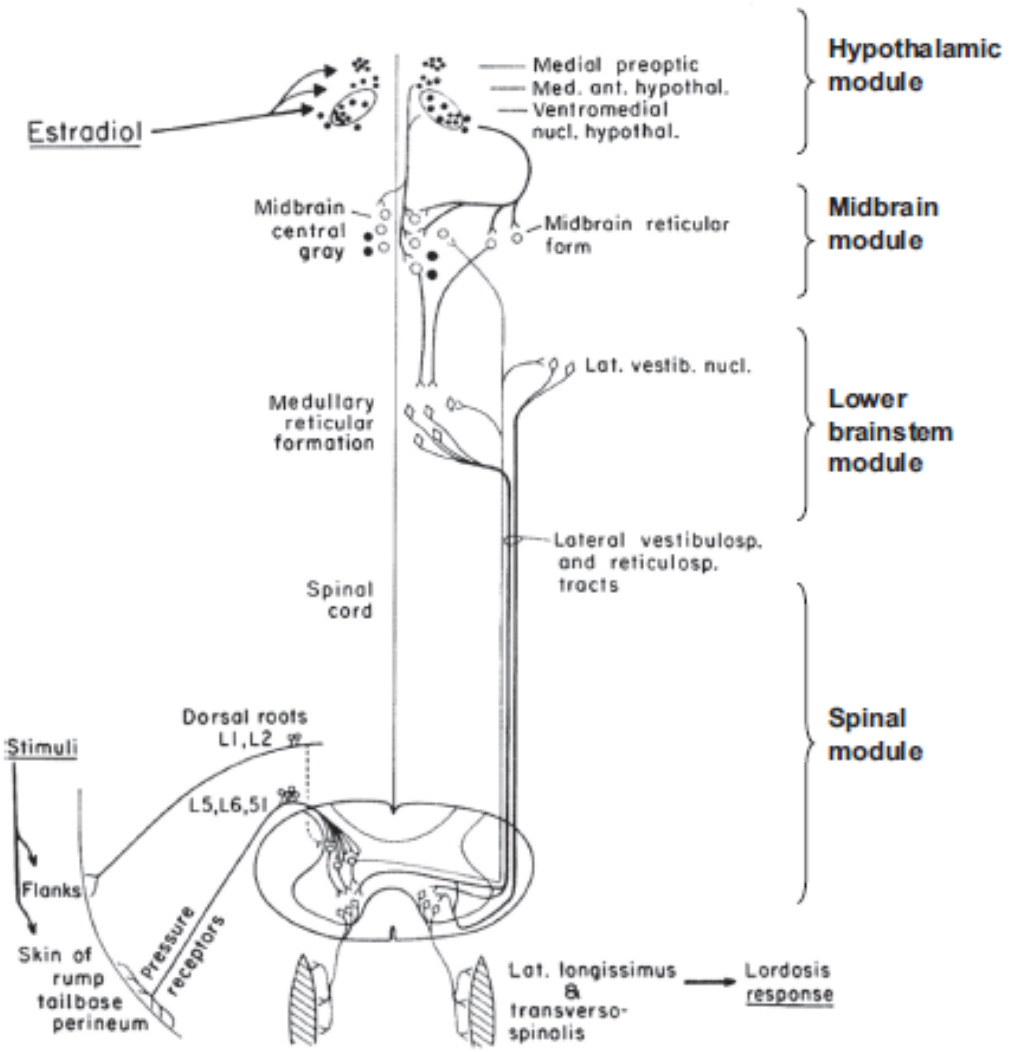

Figure 1. The neural circuitry that produces the lordosis behaviour in the rat, composed by Pfaff and colleagues (1980; 2008). This circuit consists of four modules that act as centres of control underlying different functions required to display lordosis behaviour. (Adapted from Pfaff, Kow, Loose, & Flanagan-Cato, 2008. Reprinted with permission from Elsevier).

The *midbrain module* consists primarily of the medullary reticular formation (MRF). This module integrates the motor output necessary across spinal segments, coordinating the animal and allowing for it to remain upright via projections to the spinal module (Pfaff, 1980). The midbrain module connects the hindbrain module with the uppermost module (the hypothalamic module) by translating the endocrine message provided by the hypothalamic module into central nervous system signaling. As conceptualized by Pfaff (1980), this module centres around the central gray (also referred to as the periaqueductal gray; PAG), which plays an important, albeit not fully understood, role in lordosis (e.g., Sakuma & Pfaff, 1979a; Sakuma & Pfaff, 1979b). Collectively, it appears that genitosensory information received from the male during mounts with pelvic thrusting and intromission activates afferent inputs to the lateral PAG, which in turn projects to the MRF, allowing lordosis when the hormonal circumstances are appropriate. When they are not, defensive behaviours and/or aggressive rejections of the male are exhibited (Barnett, 1963; Pfaus et al., 2015). This suggests that lordosis is under tonic inhibition for at least three days of the typically 4-day estrous cycle, with the lateral PAG playing a role in exerting this inhibitory control (Veening, Coolen, & Gerrits, 2014; Yamada & Kawata, 2014).

The final module in this conceptualization is the *hypothalamic module.* The main function of this module is to provide endocrine regulation in order to synchronize sexual behaviour with ovulation. This maximizes the chances of fertilization, subsequently preparing the female for pregnancy, parturition, while coordinating these phases with nutritional status and circadian signals (Pfaff et al., 2008). To release the tonic inhibition over lordosis, the hypothalamic module conveys messages down to the PAG in the midbrain module. This in turn projects to the MRF in the lower brainstem module, which then sends the communication finally down to the dorsal axial muscles via the spinal module. In Pfaff's original conception, the hypothalamic module focused predominantly on the VMH, which sends projections to the PAG (Krieger, Conrad, & Pfaff, 1979; Beitz, 1982; Morrell & Pfaff, 1982; Morrell, Schwanzel-Fukuda, Fahrbach, & Pfaff, 1984; Dornan, Akesson, & Micevych, 1990; Hennessey, Camak, Gordon, & Edwards, 1990; Akesson, Ulibarri, & Truitt, 1994; Marson, 1995; Papka, Williams, Miller, Copelin, & Puri, 1998; Daniels, Miselis, & Flanagan-Cato, 1999). Studies have consistently confirmed its critical role in controlling lordosis (Mathews & Edwards, 1977; Pfaff & Sakuma, 1979a; Pfaff & Sakuma, 1979b; Calizo & Flanagan-Cato, 2002; Flanagan-Cato,

2011). For example, lordosis is inhibited following bilateral electrolytic lesions (Pfaff, 1980) and knife transections (Mathews & Edwards, 1977; Pfaff & Sakuma, 1979b; Pfeifle et al., 1980) of this area, and an enhancement of lordosis is observed following electrical stimulation (Pfaff & Sakuma, 1979a).

The display of the lordosis reflex is only appropriate when the female is in ovulation, a timing determined in part by the sequential actions of estradiol and progesterone. The secretions of these hormones form a cascade of genomic and non-genomic actions, modifying multiple connections within the lordosis circuit. This results in both disinhibiting and directly exciting lordosis (for a review see McEwen, Davis, Parsons, & Pfaff, 1979; Pfaus et al., 2015). Estrogen is necessary for the display of the lordosis, and the VMH is the main site of action (Mathews & Edwards, 1977; Rubin & Barfield, 1983). Estradiol has many neurochemical targets for stimulating lordosis, acting to upregulate PR, oxytocin (OT) receptors, GABA-A receptors, DA D1R, GnRH receptors, delta opioid receptors, MC3 and MC4 receptors, adrenergic α1 receptors, and muscarinic receptors (de Kloet, Voorhuis, Boschma, & Elands, 1986; Schumacher, Coirini, Johnson, Flanagan, Frankfurt, Pfaff, & McEwen, 1993; Kow, Mobbs, & Pfaff, 1994; Bale & Dorsa, 1995; Griffin, Ferri-Kolwicz, Reyes, Van Bockstaele, & Flanagan-Cato, 2010; Pfaus et al., 2015). In addition, estradiol acts on DA, GABA, glutamate, GnRH, and pro-opiomelanocortin (POMC) neurons in promoting lordosis (as reviewed by Pfaus et al., 2015). Estradiol also acts in the PAG, which has an abundance of ERα (Shughrue et al., 1997; VanderHorst, Schasfoort, Meijer, van Leeuwen, & Holstege, 1998), to reorganize synaptic connections (Chung, Pfaff, & Cohen, 1988). In addition to estradiol, progesterone increases lordosis reflex magnitudes when administered to the VMH of OVX rats primed with low doses of estradiol (DeBold & Malsbury, 1989; Pleim et al., 1991; Frye & Vongher, 1999).

Glutamate transmission in the VMH inhibits lordosis. Infusions of it, or any of its agonists (i.e., kainic acid, AMPA, and NMDA), decrease lordosis (Kow et al., 1985; McCarthy, Curran, & Feder, 1991; Georgescu & Pfaus, 2006a). Conversely, infusions of different ionotropic glutamate antagonists (e.g., AP-5, CNQX, and DNQX) to the VMH increases lordosis (Georgescu & Pfaus, 2006b). Consistently, studies have shown that ovarian hormones alter glutamate transmission in the VMH (e.g., Kow et al., 1985; Schumacher et al., 1989; McCarthy, 1995). An early HPLC study determined that systemic estradiol treatment increases glutamate levels in the VMH, whereas subsequent progesterone treatment reverses this effect (Luine,

Grattan, & Selmanoff, 1997). However, a more recent study utilizing microdialysis followed by HPLC, and using a slightly larger dose of EB (10 μg rather than 5 μg), reported that EB treatment, with or without P, decreased glutamate release in the VMH in response to presentation of a male (Georgescu et al., 2014). Similarly, glutamate neuronal activation has been shown to decrease following EB treatment with or without P (Georgescu et al., 2009). Receptor subtypes such as AMPA and NMDA are upregulated following estradiol treatment as well (Diano, Naftolin, & Horvath, 1997; Watanabe, Inoue, Hiroi, Orimo, & Muramatsu, 1999). Glutamate neurons in the VMH express ERα (Eyigor, Lin, & Jennes, 2004). Thus, lordosis is dependent, in part, on the inhibition of glutamate neurons in the VMH. This effect could occur in combination with OT, which is co-released with glutamate in the VMH and could help signal social and sexual cues (Pfaus et al., 2015), or via estradiol activation of GAD, the enzyme that converts glutamate to GABA, thereby altering the functional phenotype of these neurons from one that inhibits lordosis to one that disinhibits it.

Dopamine is another neurotransmitter that facilitates lordosis, as infusions of DAR agonists to the VMH increase lordosis (Foreman & Moss, 1979; Apostolakis, Garai, Fox, Smith, Watson, Clark, & O'Malley, 1996), whereas infusions of DAR antagonists decrease this behaviour (Foreman & Moss, 1979). This effect appears to be driven by the DA D1R subtype, specifically through DA D5R (Apostolakis et al., 1996). Further work by Mani and colleagues indicates that this stimulatory effect may be due to activation of DA D1R in the VMH resulting in a ligand-independent activation of PRs (e.g., Mani et al., 1994; Mani et al., 1996; Mani et al., 2000; Mani et al., 2001).

As alluded to above, the function of GABA in the VMH is facilitative, with GABA-A agonists promoting lordosis likely via disinhibition (McCarthy, Masters, Fiber, Lopez-Colombe, Beyer, Komisaruk, & Feder, 1991; McCarthy, 1995; Luine, Wu, Hoffman, & Renner, 1999). This effect could occur by GABA inhibiting the glutamatergic interneurons that are inhibiting lordosis in this region, or by the estradiol-induction of GAD within glutamate neurons, thus changing them from glutamate to GABA neurons and making their influence disinhibitory. It has also been theorized that the effect of GABA may also act by inhibiting serotonin in the VMH (Luine et al., 1997; Hoffman, Westin, Miner, Johnson, Summers, & Renner, 2002). Regardless of the exact mechanism, lordosis is increased following infusions of muscimol, a GABA-A receptor agonist, into the VMH (McCarthy, Malik, & Feder, 1990; Hoffman et al., 2002), but

decreased following infusions of bicuculline, a GABA-A receptor antagonist (McCarthy et al., 1990). Estradiol modulates GABA in the VMH (Kow et al., 1985; Schumacher et al., 1989; McCarthy, 1995), with E2 treatment increasing the levels and P administration returning them back to vehicle baseline (Luine et al., 1997). Thus, the action of GABA-A receptors on an unknown inhibitory substrate in the VMH facilitates lordosis.

The Odd Role of the mPOA in Lordosis

The medial preoptic area is a unique region of the hypothalamus that plays an imperative role in the control of a variety of regulatory behaviours. These include the hormonal control of motivational aspects of sexual behaviour in both males and females (Pfaus, 2009; Pfaus et al., 2010). Although included in the aforementioned lordosis circuit by Pfaff and his colleagues (e.g., Pfaff, 1980; Pfaff et al., 2008), the role of the mPOA in female rat sexual behaviours has largely been downplayed, as early studies indicated it played an inhibitory role (e.g., electrical stimulation of this area decreased lordosis; Napoli et al., 1972; Moss et al., 1974; Pfaff & Sakuma, 1979a; Takeo et al., 1993, whereas lesions to the mPOA enhanced lordosis; Law & Meagher, 1958; Powers & Valenstein, 1972; Clemens et al., 1976; Kondo, Shinoda, Yamanouchi, & Arai, 1990; Olster, 1993; Takeo et al., 1993; Hoshina et al., 1994; Olster & Paulson, 1997; Xiao, Kondo, & Sakuma, 2005). There is some debate however whether the potentiation of lordosis by mPOA lesion is context-specific. For example, Whitney (1986) reported that these effects depend on the specific testing situation utilized. When a female is tested in an environment she cannot escape, meaning the female is less able to control the amount of stimulation she receives from the male, lordosis is potentiated following mPOA lesions. However, when tested in an environment she can escape, allowing her to determine her preferred intervals of VCS, the display of lordosis was not altered (Whitney, 1986). Precopulatory behaviour was differentially affected in both of these scenarios, suggesting a more complex role for the mPOA in female sexual behaviour. This presumably provides flexibility to a female's strategy of copulation under different environmental circumstances. As elaborated upon below, this strategy may allow for the female to properly time copulatory responses controlling the frequency and intensity of male contact to maximize reward (Paredes & Vazquez, 1999).

Neural activation is also seen in the mPOA following copulation and related clitoral and vaginocervical stimulation (Wersinger, Baum, & Erskine, 1993; Pfaus, Jakob, Kleopoulos, Gibbs, & Pfaff, 1994; Flanagan-Cato & McEwen, 1995; Oboh, Paredes, & Baum, 1995; Polston & Erskine, 1995; Coolen, Peters, & Veening, 1996; Yang & Voogt, 2002; Cameron & Erskine, 2003; Pfaus, Manitt, & Coopersmith, 2006; Coria-Avila & Pfaus, 2007; Parada, Chamas, Censi, Coria-Avila, & Pfaus, 2010; Kirkpatrick & Merrill, 2011). The role of the mPOA in lordosis may well be due to its reciprocal connections with the VMH, and in particular the ventrolateral VMH (VMHvl; Saper, Swanson, & Cowan, 1976; Chiba & Murata, 1985; Simerly & Swanson, 1986; Bast, Hunts, Renner, Morris, & Quadagno, 1987; Simerly & Swanson, 1988). It should be noted however that some older studies found no change in lordosis following alteration of the mPOA-VMH connection (e.g., Singer, 1968; Numan, 1974; Gray et al., 1978). Alternatively, or in conjunction, the mPOA may act via the PAG (Chiba & Murata, 1985; Simerly & Swanson, 1988; Rizvi, Ennis, & Shipley, 1992), as evidence exists for activation of GnRH neurons following sexual stimulation (Wu, Segal, Miller, Gibson, & Silverman, 1992; Pfaus et al., 1994). These neurons project from the mPOA and are hypothesized to be involved in communication with the PAG (Veening et al., 2014). The mPOA also sends a larger output through the PAG directly to the nPGi (Marson & Foley, 2004). Both sets of these mPOA efferents terminate on neurons that project to the spine, indicating pathways through which the mPOA regulate genital reflexes (Marson & Foley, 2004) (see Figure 2).

The findings from Chapter 4 of the current thesis indicate that administering EB increases the proportion of neurons projecting from the mPOA to the VMH that are GABAergic, and this finding is irrespective of concurrent administration of P (see Figure 3). Additionally, this increase is accompanied by a greater proportion of DA D2R located on these projection neurons. If P is administered after EB, an increase of DA D1R is seen instead on these GABA neurons. This aligns with behavioural studies indicating that precopulatory behaviours are increased in EB-alone females following activation of DA D2R, and in EB+P females following DA D1R activation (Graham & Pfaus, 2010; Graham & Pfaus, 2012). GABA in the VMH facilitates lordosis by a presumably disinhibitory action (McCarthy, 1995; Luine et al., 1999). Given the present findings, it is possible that an increase in GABAergic transmission from the mPOA to the VMH may be responsible for promoting precopulatory behaviours, acting to "shut off" this

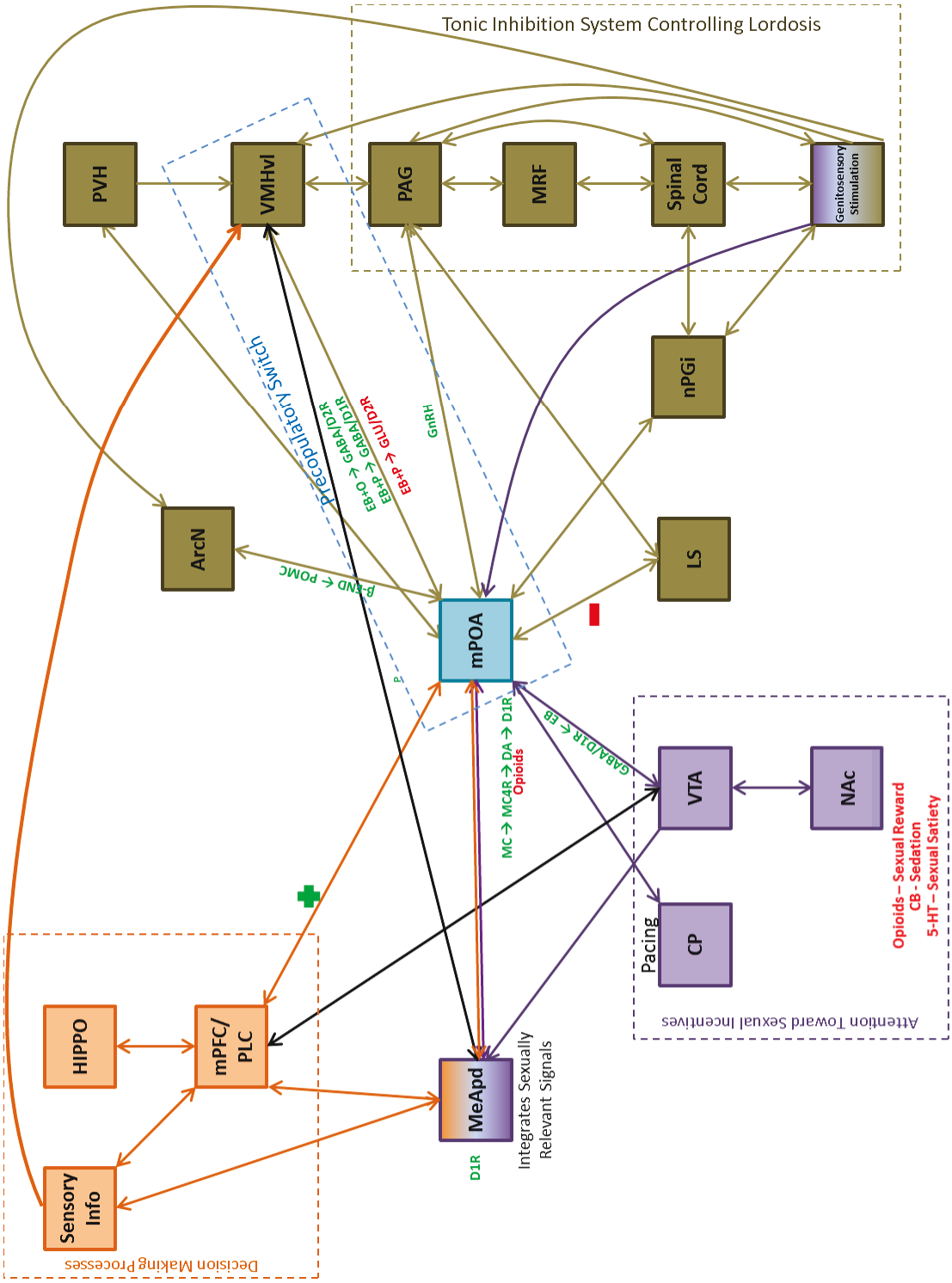

Figure 2. Conceptual model showing neural circuitry projecting to and from the mPOA in the control of female sexual behaviour.

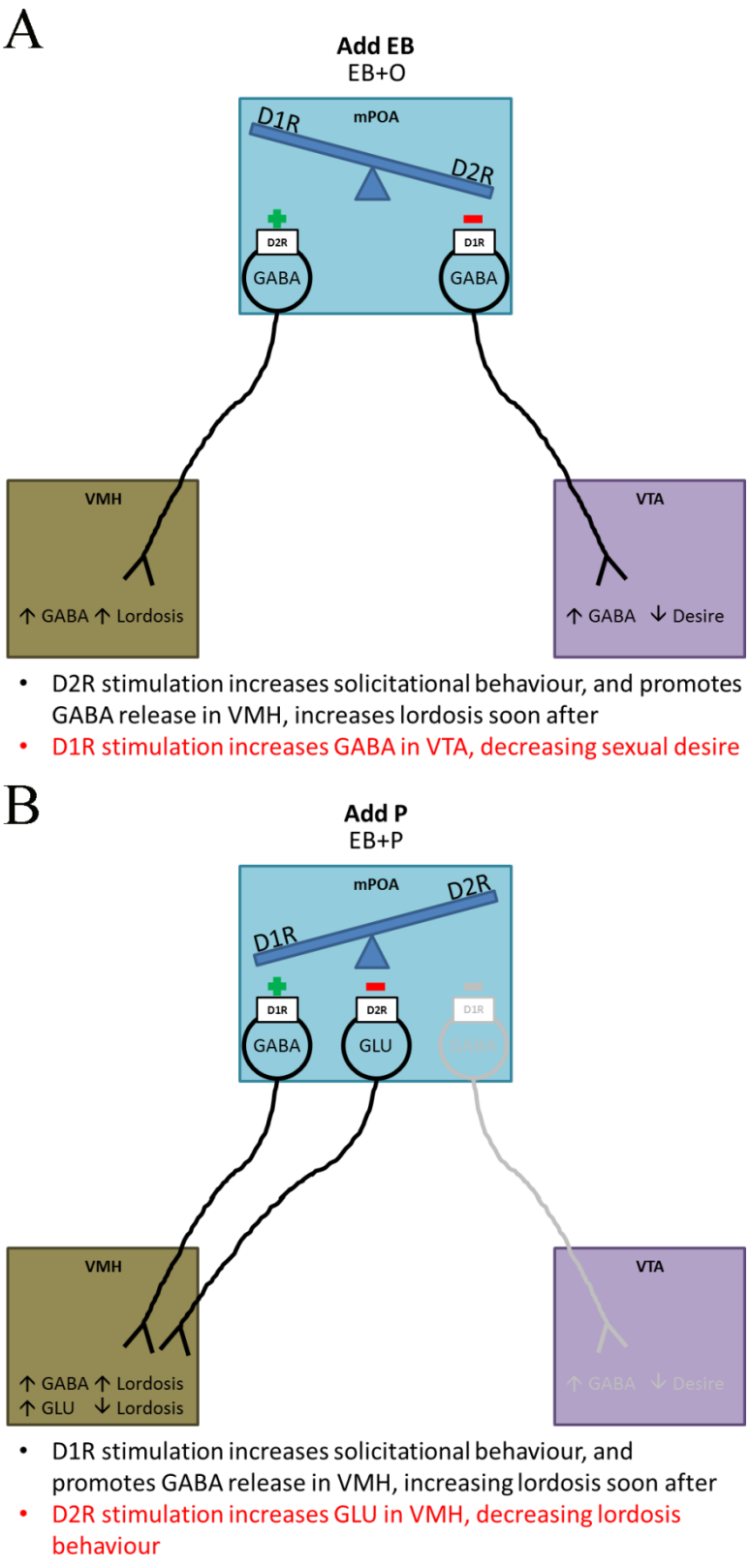

Figure 3. Schematic diagrams of neurobiological changes in the projections from the mPOA to the VMH and VTA under (A) EB-alone and (B) EB+P hormonal conditions.

inhibitory system. This may be accomplished via the corresponding DAR-system that is seen under the two hormonal conditions, observed behaviourally (Graham & Pfaus, 2010; Graham & Pfaus, 2012) and as confirmed in Chapter 3. Under this scenario, when only EB is circulating and thus the animal is in a low receptive state, the increase of DA D2R on GABAergic neurons in the mPOA projecting to the VMH help promote the release of GABA there to disinhibit lordosis. This would increase the display of consummatory behaviours. When P is added, so that the animal is fully-primed and highly receptive, an increase of DA D1R on GABAergic projection neurons from the mPOA to the VMH instead act to release GABA in the VMH, thereby increasing lordosis through disinhibition.

Furthermore, when females are fully-primed hormonally with EB+P, the efferents from the mPOA to the VMH are more often glutamatergic, and are more likely to have DA D2R on them (see Figure 3). Glutamate in the VMH decreases precopulatory behaviours (Georgescu & Pfaus, 2006a; Georgescu & Pfaus, 2006b) and lordosis (Kow et al., 1985; Georgescu & Pfaus, 2006a; Georgescu & Pfaus, 2006b), and the findings of Chapter 4 indicate this is another system from which the mPOA may help control female sexual behaviour, and in particular precopulatory behaviours. Specifically, when females are fully sexually responsive, an increase in glutamatergic connections from the mPOA to the VMH can act as an "off" switch to decrease precopulatory behaviours. This would occur via DA D2R, corresponding to our previously discovered behavioural data (Graham & Pfaus, 2012), and again confirmed by the studies of Chapter 3. Taken together, the connections of the mPOA to the VMH are altered under different hormonal manipulations and are potentially responsible in helping control female sexual behaviour. In particular, these connections could control the transition from precopulatory to consummatory behaviours, as observed under stereotypical copulatory bouts (see below).

Another key brain region in the control of female sexual behaviour, the arcuate nucleus (ArcN), also relies on connections to the mPOA to exert its control. The ArcN has shown neural activation following genitosensory stimulation (Erskine, 1993; Parada et al., 2010), and tracing studies have determined it receives input from the ovaries (Gerendai, Toth, Boldogkoi, Medveczky, & Halasz, 1998). POMC is a precursor peptide largely made in the ArcN, and from it many important neuromodulatory substances are derived. These include melanocortins such as α-MSH, the opioid β-endorphin, and ACTH (Cooper & Martin, 1980). Estradiol stimulates

neurons in the ArcN to synthesize POMC (Taylor, Goubillon, Broad, & Robinson, 2007), as well as α-MSH (Khorram, Bedran de Castro, & McCann, 1985).

The ArcN has been suggested to be the starting point for estradiol to activate the limbic-hypothalamic circuit controlling lordosis (Mills, Sohn, & Micevych, 2004; Dewing et al., 2007). The hypothesis is that through signalling by a biotinylated form of estradiol, specially designed to be impermeable to the cell, a microcircuit is stimulated involving neuropeptide Y (NPY)-innervating, β-endorphin-expressing, POMC neurons in the ArcN. Through an interaction between membrane-associated estrogen receptor-alpha (mERα) and the metabotropic glutamate receptor 1a (mGluR1a), an activation of NPY-Y1 receptors results, and release of β-endorphin in the mPOA is caused (Sinchak, Roselli, & Clemens, 1996; Mills et al., 2004). This chain reaction acts on μ-opioid receptor (MOR)-containing neurons in the mPOA that project to the VMH, internalizing them and causing a transient inhibition that is required for the display of lordosis (Eckersell, Popper, & Micevych, 1988; Torii, Kubo, & Sasaki, 1996; Torii, Kubo, & Sasaki, 1999; Sinchak & Micevych, 2001; Mills et al., 2004; Sinchak, Shahedi, Dewing, & Micevych, 2005). This effect has been shown to respond to both EB-alone and EB+P conditions (Mills et al., 2004; Dewing et al., 2007). For example, an increase of E2 causes an internalization of MOR in the mPOA, and a further increase of P reverses this internalization. This has been suggested as possibly being responsible for the timing mechanism of inducing sexual behaviour to coincide with ovulation (Sinchak & Micevych, 2001; Acosta-Martinez & Etgen, 2002).

The mPOA is also connected to the LS, a region thought to play a prominent role in suppressing lordosis (Nance, Shryne, Gordon, & Gorski, 1977; Vomachka, Richards, & Lisk, 1982; King & Nance, 1985; Kondo et al., 1990; Kondo, Koizumi, Arai, Kakeyama, & Yamanouchi, 1993; Yamanouchi & Arai, 1990; Floody, 1993; Tsukahara & Yamanouchi, 2001; Tsukahara & Yamanouchi, 2002; Tsukahara, Ezawa, & Yamanouchi, 2003; Xiao et al., 2005; Segovia, Garcia-Falgueras, Perez-Laso, Pinos, Carrillo, Collado, Claro, & Guillamon, 2009). Known mostly for its role in suppressing aggression (Albert & Richmond, 1975; Haller, Toth, Halasz, & De Boer, 2006; Dhakar, Rich, Reno, Lee, & Caldwell, 2012), the LS is thought to also counteract the facilitative role of the VMHvl in lordosis (Segovia et al., 2009). No clear evidence supports the LS directly affecting the VMH (King & Nance, 1985), though descending fibres from the LS pass through the mPOA and VMHvl (Veening, Swanson, Cowan, Nieuwenhuys, & Geeraedts, 1982; Geeraedts, Nieuwenhuys, & Veening, 1990), ending in the

PAG (Yamanouchi & Arai, 1990; Tsukahara & Yamanouchi, 2002), and these fibres are sensitive to estrogen (Tsukahara & Yamanouchi, 2002; Tsukahara et al., 2003). This indicates that effects of the LS are likely both via direct PAG connections and supported by the lordosis-inhibiting effects of the mPOA (Floody & DeBold, 2004; Xiao et al., 2005).

Sexual Reward Circuit Background

Although sensory information and lordosis are required for a sexual response from a female, the female must be motivated to display and engage in such behaviours. To infer sexual motivation in non-human animals, behaviours that denote sexual desire must be determined and observed. The strength of certain female sexual behaviours can be used to infer sexual motivation or desire, including the number of solicitations, her pacing behaviour, and lordosis magnitude (Pfaus et al., 2010; Pfaus et al., 2015). Typically, solicitations and pacing occur prior to the receipt of sexual stimulation by the male. Females can however also display behaviours inferring sexual desire from precopulatory behaviours in between copulatory bouts. Additionally, laboratory procedures can measure sexual desire through the measurement of operant responses, time spent near cues indicating sexual incentives, or conditioned locomotor responses (Pfaus, 2009; Pfaus et al., 2010).

Pfaus, Ismail, and Coria-Avila (2010) constructed a sexual reward circuitry based on the display of female sexual behaviours in response to conditioned olfactory cues that direct their partner preferences. The sexual reward circuit involves at least three neurochemical systems (Pfaus et al., 2015) that focus the attention of the female and allow her to engage in forward-directed movements towards the appropriate sexual incentive stimulus when she is in the vicinity (Robinson & Berridge, 1993; Berridge, Robinson, & Aldridge, 2009). These systems include the mesolimbic DA system, hypothalamic OT, and opioids (Pfaus et al., 2015). The limbic system is an integral aspect of this circuitry, especially the mesolimbic DA system. Its actions are typically initiated by ovarian steroids, with continuous activation during copulatory interaction, and sensitization by opioids during periods of sexual reward (in which females display orgasm-like responses; Pfaus et al., 2015). Sexual reward, in turn, enhances phenomena such as conditioned place or partner preference, and steroid hormones themselves influence mating-induced preferences (Corona, Camacho, Garcia-Horsman, Guerrero, Ogando, & Paredes, 2011; Parada, Vargas, Kyres, Burnside, & Pfaus, 2012).

Systems within the sexual reward circuit are organized to have excitatory and inhibitory components that are presumably interacting constantly. The excitatory system consists of mesolimbic, nigrostriatal, and hypothalamic DA pathways that operate to focus attention towards external incentive stimuli and for the activation of specific sexual responses. It is also comprised of noradrenergic pathways underlying central and autonomic arousal. The mesolimbic DA system as a whole is responsible for the sensitization and crystallization of incentive responding (Robinson & Berridge, 1993). Projections from the mesolimbic DA system go to various structures of the limbic system and cortex, such as the nucleus accumbens (NAc), MeA, lateral septum (LS), and mPFC, helping to coordinate decision making with additional outputs responsible for motor activity, such as the caudate putamen (CP) and the ventral pallidum. In particular, activation of DA neurons in the VTA is important to stimulate DA release in the NAc. This mechanism underlies the increased attention toward sexual incentives during the appetitive and precopulatory phases of sexual behaviour. This ensures that appetitive motivation is focused on appropriate sexual partners acting as rewarding incentives. In support of this, neural activation has been observed in both the VTA (Coria-Avila & Pfaus, 2007) and the NAc (Parada et al., 2010; Pitchers, Frohmader, Vialou, Mouzon, Nestler, Lehman, & Coolen, 2010; Klingerman, Patel, Hedges, Meisel, & Schneider, 2011; Parada et al., 2012; Pitchers, Schmid, Di Sebastiano, Wang, Laviolette, Lehman, & Coolen, 2012) in association with sexual reward, while females that receive NAc lesions avoid sexual contact (Rivas & Mir, 1990). Additionally, increases of DA in the NAc are seen during copulation of fully hormonally-primed females (Pfaus, Damsma, Wenkstern, & Fibiger, 1995), and when hormonally-primed females have to perform operant responses to gain access to males (Jenkins & Becker, 2003).

For female rats, one of the main ways they maximize pleasure from the sexual stimulation they receive is to control or "pace" the initiation and rate of that stimulation. Pacing behaviour thus regulates the timing of copulatory stimulation, increasing both the reward and reproductive value of the stimulation (Paredes & Alonso, 1997; Paredes & Vazquez, 1999; Martinez & Paredes, 2001). DA release in the NAc serves to orient the female towards an appropriate sexual stimulus (i.e., a sexually active male). In response to this, it is thought that DA release in the striatum, such as the CP, produces a transition for the female to then make approach responses to this stimulus. Striatal DA is thus hypothesized to enhance motor output overall, but especially pacing behaviour (Becker, 1999). Lesioning the CP results in females

showing a reduction of pacing sexual encounters, as animals were less likely to leave the male. Lesioning the NAc, conversely, results in the females avoiding sexual contact with the male altogether (Jenkins & Becker, 2001).

DA is known to play a role in the CP during female copulation (e.g., Pfaus et al., 1995; Becker et al., 2001), and to interact with estrogen in doing so (e.g., Mermelstein & Becker, 1995; Meredith et al., 1997). Estrogen has been shown to affect DA and its receptors in the rat striatum in various ways, such as altering receptor densities and binding affinities, and modifying DA release. This occurs both naturally through fluctuation over the estrous cycle (Di Paolo et al., 1988; Levesque et al., 1989), as well as via administration to OVX females (for review, see Hruska, 1985; Becker, 1999). No effects have been found in altering DAR density in women across the menstrual cycle (Nordström, Olsson, & Halldin, 1998).

Most studies examining the effect of estrogen on DA focus on repeated estrogen administration (e.g., Levesque & Di Paolo, 1989; Levesque & Di Paolo, 1991; Levesque & Di Paolo, 1993; Lammers et al., 1999), and the ones that gave acute injections typically used adult male rats (Hruska, 1986; Hruska & Nowak, 1988). Alternatively, administered doses and timing schedules were used that fell outside of the normal sexual priming methods commonly practiced (Di Paolo et al., 1985; Levesque & Di Paolo, 1988; and reviewed in Hruska, 1985; Becker 1999; Sanchez et al., 2010). The effect of P in combination with EB administration on DA in the CP has received much less attention. When given independently, P was found to decrease DA D2R four hours later, but this effect was not seen when EB was given prior. However there was an increase in DA D2R levels 24 hours after EB+P treatment (Fernandez-Ruiz et al., 1989).

In concordance with these previous studies, the studies of the present thesis, as seen in Chapter 3, failed to find an effect of acute hormone administration on either DAR subtype in the CP following either fully or partially sexual-priming hormonal regimens. Any effect of steroid hormones on DAR in the CP therefore occurs either much earlier than when females are most sexually receptive, or much later (Gordon, 1980; Gordon & Perry, 1983; Fernandez-Ruiz et al., 1989). Any behavioural effects will presumably be due to DAergic changes not pertaining to the receptors themselves.

The Role of the mPOA in Sexual Reward

The mPOA is also critical for the experience of sexual reward in both males and females. Neural activation associated with female sexual reward has been found in the mPOA (Coria-Avila & Pfaus, 2007), and clitoral stimulation activates Fos in the mPOA (Parada et al., 2010), with clitoral afferents projecting to this area (Marson, 1995). Lesions of the mPOA result in increased withdrawals from the male following intromissions, and increase the latencies to return to the male following intromissions and ejaculations (Guarraci, Megroz, & Clark, 2004). This suggests that keeping females in the vicinity of males is a role for mPOA activation. Finally, lesions of the mPOA also disrupt VCS-induced reward (Meerts & Clark, 2009), again indicating that the mPOA is an important area in the receipt of sexual reward for the female. It should be noted however, that not all pacing research has shown a consistent effect of the mPOA, as female exits were not disrupted following radiofrequency lesions of the area (Xiao et al., 2005).

The mPOA is also connected to the sexual reward system, as it sends efferents to areas of the mesolimbic system, in particular the VTA (Simerly & Swanson, 1988; Zahm et al., 2011). This has long been incorporated in models pertaining to male sexual behaviour in the rat (e.g., van Furth, Wolterink, & van Ree, 1995), but has been neglected in female sexual behaviour. For example, although the neurobiology of pair bonding is well understood in prairie voles, the model by Young and Wang (2004) fails to show a role for an mPOA-VTA projection in activating the sexual reward circuit. This connection is known to exist in rats, and serves to stimulate DA neurons, which make up approximately 65% of the neurons in the VTA (Margolis, Lock, Hjelmstad, & Fields, 2006; Nair-Roberts et al., 2008). Thus DA release is promoted in areas of the mesolimbic and mesocortical terminals, such as the NAc, MeA, LS, anterior cingulate cortex, and mPFC (Fallon & Moore, 1978; Swanson, 1982; Björklund & Lindvall, 1984; Phillipson & Griffiths, 1985; Robinson & Berridge, 1993). Activation of DA neurons in the VTA is controlled by excitatory input acting via N-methyl-D-asparate (NMDA) and non-NMDA receptors, inhibitory input acting via GABA-A and GABA-B receptors, and through DAergic input primarily on DA D2R (Johnson & North, 1992). Recently it has been discovered that some of the GABA entering the VTA originates in the mPOA, and that these neurons contain DAR (Tobiansky et al., 2013).

The findings from Chapter 4 of the current thesis indicate that administering EB, with or without P, decreases the proportion of neurons projecting from the mPOA to the VTA that are

GABAergic and have DA D1R on them (see Figure 3). Since DAergic activity in the VTA is inhibited by GABA-A and GABA-B receptors (Johnson & North, 1992), perhaps this alteration in the mPOA-VTA connection increases the female's sensitivity to sexual reward. This may be one mechanism promoting female sexual behaviour in response to an increase in EB. Alternatively, or additionally, this mechanism perhaps reduces GABA-mediated inhibition, allowing for the VTA to facilitate lordosis (Frye, 2001; Frye, 2011).

Other projections of the mPOA could exert control over female sexual behaviour, including projections to the medial amygdala (MeA), and in particular its posterior-dorsal aspect (MeApd; Canteras, Simerly, & Swanson, 1995). This would give the mPOA an integrative role in connecting paced copulation and sensory signals to sexual reward. The mPOA also has a large efferent output to the paraventricular nucleus (PVN) (Saphier & Feldman, 1986), and OT is released from this area. Infusion of OT, important in bonding (Carter, Williams, Witt, & Insel, 1992; Young & Wang, 2004), and infusions to the mPOA increases lordosis (Caldwell, Jirikowski, Greer, & Pedersen, 1989; Schulze & Gorzalka, 1991). The PVN has been found to stimulate neural activation in response to sexual activity (Flanagan, Pfaus, Pfaff, & McEwen, 1993; Yang & Voogt, 2002; Cameron & Erskine, 2003), suggesting that a PVN-mPOA connection that utilizes OT may facilitate both lordosis and bonding.

The Role of the mPOA in Sensory Information

The mPOA has been proposed to integrate inputs necessary for the activation of both appetitive and consummatory sequences of female sexual behaviour (e.g., Pfaus et al., 2015), which include genitosensory, olfactory, and other sensory information. In particular, the mPOA receives input from the medial prefrontal cortex (mPFC) (Groenewegen & Uylings, 2000; Saper, 2000), including the PLC in particular (Balfour, Brown, Yu, & Coolen, 2006). The mPFC in turn is extensively connected with olfactory, auditory, visual, gustatory, somatosensory, and premotor cortical areas (Van Eden, Lamme, & Uylings, 1992; Condé, Maire-Lepoivre, Audinat, & Crepél, 1995; Groenewegen & Uylings, 2000).

The mPFC also shares vast connections with the hippocampus (Swanson, 1981; Gabbott, Headlam, & Busby, 2002), an area critical for episodic memory and which provides spatial maps of the external world (e.g., Burgess, Maguire, & O'Keefe, 2002), and the amygdala, which encodes attentional and emotional components as part of the larger limbic system (e.g., Baxter & Murray, 2002). Thus, the mPFC is thought to be a major integrator for decision-making

processes (Tranel, Bechara, & Denburg, 2002), and involved in social-emotional aspects of behaviour (e.g., Nauta, 1971; Damasio, Tranel, & Damasio, 1991; Price, Carmichael, & Drevets, 1996), incorporating emotional processes and previous memories to help guide the animal's decisions (Groenewegen & Uylings, 2000). Through its projections to the mPOA the mPFC may communicate imperative information for further moderation; an idea that forms the basis of the 'somatic marker' hypothesis (Damasio, 1994). The mPOA also shares important connections with the hippocampus (Olpe & McEwen, 1976; Cenquizca & Swanson, 2006) and amygdala (Canteras et al., 1995). It is possible that by incorporating the information received from the mPFC with these two areas that the mPOA integrates attentional and emotional components in conjunction with the received sensory information to coordinate behavioural output. This coordination would better guide the decisions of the female to more appropriately direct her intentions towards a sexually-stimulating male (e.g., Nauta, 1971; Damasio et al., 1991; Price et al., 1996; Groenewegen & Uylings, 2000). More information regarding the role of the mPOA in this sensory information circuit is required. Also of note is that synaptic changes caused by estradiol induction have been observed in both the hippocampus of rats (Woolley & McEwen, 1993) and the PFC of rhesus monkeys (Tang, Janssen, Hao, Roberts, McKay, Lasley, Allen, Greengard, Rapp, Kordower, Hof, & Morrison, 2004), indicating that the sensory information received and processed may be altered by ovarian hormones.

In the context of the current thesis, there has been minimal research for the role of the PLC, an area within the mPFC, on sexual behaviour, with most of the research focusing on males. It has been found that male sexual behaviour activates neurons in the PLC, as measured by Fos protein immunoreactivity, and the PLC sends projections to multiple areas important for male sexual behaviour, including the mPOA, NAc, and VTA (Balfour et al., 2006). However a later study from the same laboratory found that lesioning the PLC has no effect of the display of male sexual behaviour itself, but instead prevents the ability to acquire conditioned sexual aversion. This led to the hypothesis that this area is important in altering behaviour to fit adaptive situations (Davis, Loos, Di Sebastiano, Brown, Lehman, & Coolen, 2010).

To date no study has examined the role of the PLC on female sexual behaviour, and only one study has examined the mPFC as a whole. Afonso and colleagues (2007) found that lesioning the mPFC resulted in a decrease in precopulatory behaviours, both solicitations and hops and darts, during proestrus. In fact, solicitational sequences seemed to be particularly

affected. Lesioned females ostensibly started a solicitation with a head-wise orientation to the male, but instead of following through with a runaway, the female engaged in a different behaviour (such as hopping), breaking the solicitational pattern (Afonso, Sison, Lovic, & Fleming, 2007). This disruption tended to result in a male mount, with the female exhibiting lordosis, rather than pursuit by the male as would normally be observed. No effect of mPFC lesion on lordosis, anogenital investigations, or self-grooming was discovered (Afonso et al., 2007).

Various studies have examined ovarian hormonal effects on DA levels in the mPFC (for a review, see Shanmugan & Epperson, 2014), but few have examined how they may affect changes to DAR. It was found that DA D1R needed to be activated for estrogen to exert its effect on calcium/calmodulin-dependent protein kinase II activity in the frontal cortex for behavioural supersensitization to occur after prolonged exposure to cocaine in female rats (Zhen, Goswami, Abdali, Frankfurt, & Friedman, 2007). Feng and colleagues (2004) found that P inhibited the increase in frequency of spontaneous excitatory postsynaptic currents induced by DA, but this mechanism was not directly through interaction with DA D1R. Since P has been known to antagonize σ1 receptor, the authors hypothesized this mechanism may occur through inhibition of σ1/DA D1R synergism (Feng, Dong, Fu, Zhu, Sun, Wang, Sun, & Zheng, 2004). The results from the experiments of the current thesis, in Chapter 3, imply that DAR in the PLC may play a role in the display of female sexual behaviours, potentially by organizing the sequencing of precopulatory demonstrations. Ovarian steroid hormones may modulate these receptors in order to control the decision-making circuitry responsible for their presentation.

Additional Projections From the mPOA

One last interesting finding from Chapter 4 of the current thesis indicates that females that receive EB+P have an increased proportion of DA D2R-stained cells that are glutamatergic that do not project to either the VMH or VTA. This indicates at least one more alteration concerning the role for the mPOA in the female sexual behaviour circuitry by ovarian hormones. This pathway could include one or more of any of the previously discussed regions, including the mPOA projection to the PAG and/or nPGi (Marson & Foley, 2004) in the organization of the spinal circuitry involved in lordosis expression (e.g., Sakuma & Pfaff, 1979a; Sakuma & Pfaff, 1979b; Pfaff et al., 2004) or the inhibition of genital reflexes (e.g., Marson & McKenna, 1990; Marson et al., 1992; Marson & McKenna, 1994). This increase could be how the mPOA signals

to these areas in order to release the tonic inhibition of the urethrogenital reflex, which would result in vaginal vasocongestion (Giuliano et al., 2001).

This altered connection by ovarian hormones could also be modifying the projections to the ArcN (Conrad & Pfaff, 1975; Conrad & Pfaff, 1976). Signals could be returned to the ArcN in response to the MOR decrease and β-endorphin release in the mPOA stimulated via the ERalpha/mGluR1a interaction on NPY-Y1 receptors contained on POMC neurons in the ArcN (Sinchak et al., 1996; Mills et al., 2004). This could be another pathway through which the mPOA helps to act as a timing mechanism, sending feedback to the ArcN to "turn off" the MOR decrease resulting in the transient inhibition required for the display of lordosis (Eckersell et al., 1988; Torii et al., 1996; Torii et al., 1999; Sinchak & Micevych, 2001; Mills et al., 2004; Sinchak et al., 2005).

Another way the mPOA could affect lordosis is through its connection with the LS. The unknown alterations to the connections found in Chapter 4 could be projecting from the mPOA to the LS in order to communicate and coordinate behavioural output with this area. Under EB+P conditions, the increase in proportion of DA D2R-stained cells that are glutamatergic could act as an "off switch" indicating to the LS that it is time for disinhibition, allowing the lordosis display to occur. A mechanism between the LS and the mPOA has previously been hypothesized (Floody & DeBold, 2004; Xiao et al., 2005), and is an interesting possibility for at least one potential method regarding how the mPOA helps coordinate the switch between precopulatory and consummatory behaviours in the female rat.

The DA D2R-containing glutamatergic neurons projecting from the mPOA that were modified under EB+P conditions could also affect female sexual behaviour in ways that do not include lordosis. They could be involved in communicating with areas important for receiving sensory information (e.g., mPFC, hippocampus, amygdala), allowing for the coordination of behavioural output by the mPOA by influencing global sensory information coordination, attentional components, and/or emotional components, respectively. This may allow for adaptations by the female to new incoming sensory information, which might let the female alter her decision-making towards different sexual stimulation. A key component of these decisions might be an increase in bonding, through mPOA-PVN connections, and this is another connection that may be modified under these circumstances. Perhaps the mPOA-PVN

connection is a way to facilitate bonding behaviour and lordosis concurrently, after the mPOA has coordinated previous sensory information.

A final important connection that may be modified by EB+P hormone administration could contribute to the role of the mPOA in female sexual reward, as the mPOA shares connections with the MeA (Canteras et al., 1995). The MeA, and the MeApd in particular, has been hypothesized to process and integrate sexually relevant signals (e.g., Erskine, 1993; Pfaus et al., 1993), and thus helps in the decision making process of when motivated sexual behaviours should be displayed (Holder, Veichweg, & Mong, 2015). Perhaps the MeApd communicates with the mPOA in order to modify lordosis and precopulatory outputs towards properly focused targets. Additionally the mPOA, in its proposed function as an integration area, could serve to receive and send information regarding paced mating, in addition to the previously discussed sensory information, in order to again modify lordosis and precopulatory outputs. This pacing behaviour is imperative for spacing out male stimulation. A requirement of this is the control of when to signal the female's readiness and acceptance for a mount, through precopulatory behaviours; when to receive intromissions, through lordosis; and when sexual contact is undesirable, through escapes and/or aggression. Thus the mPOA, in connection with the MeApd and perhaps other areas, may act to control excitation and inhibition in order to time the presentations of these various displays.

Any or all of these areas could have their projections modified through the administration of female sexual hormones. By doing so, the readiness and capability of the female to display appropriate behaviours targeted at appropriate sexual stimuli can be enhanced. More research of what these connections may be, and how they ultimately affect the sexual behaviour of the female, should be performed. Doing so will only help to clarify the complexity of the female sexual behaviour circuitry, and how ovarian hormones maximize the utility of it.

The Role of the mPOA in Precopulatory Behaviours

In addition to being a central area for the integration of different circuitries for female sexual behaviour, the mPOA itself may play a direct role in precopulatory behaviours. Lesions of the mPOA have been shown to abolish precopulatory behaviours (Hoshina et al., 1994; Guarraci et al., 2004), as well as the mounting the female performs when trying to induce a sexual response from a sexually sluggish male (Afonso, Lehmann, Tse, Woehrling, & Pfaus, 2009). These data are consistent with the original findings by Whitney (1986), and noted by

Erskine (1989), that the responsiveness of mPOA lesions may be due to the characteristics of the testing environment. Thus, this may provide more evidence that the mPOA may play an integration role in regards to female sexual behaviour, and examinations of specific behaviours are context-dependent.

Several neurotransmitters have been analyzed for their effects within the mPOA on precopulatory behaviours. POMC projections from the ArcN to the mPOA secrete α-MSH. Since the mPOA contains MC4 receptors (Gelez, Poirier, Facchinetti, Allers, Wayman, Bernabe, Alexandre, & Giuliano, 2010), MC agonists, such as MT-II and bremelanotide, increase solicitations when infused into the mPOA. This effect is not found in the VMH (Pfaus, Giuliano, & Gelez, 2007). Infusions of OT antagonists induce fighting and rejection of males (Caldwell, Johns, Faggin, Senger, & Pederson, 1994), while infusions of 8-OH-DPAT resulted in an increased resistance by the female towards the male's attempt to mount (Uphouse & Caldarola-Pastuszka, 1993). Furthermore, most of the females continued showing precopulatory behaviours, with some in fact showing "frenzied" hoping and darting sequences. These results seem to be independent of the effects on male resistance, signifying that separate systems perhaps underlie precopulatory and copulatory behaviours (Uphouse & Caldarola-Pastuszka, 1993). Lastly, ascorbic acid infused into the mPOA increases precopulatory behaviours in rats primed either with EB+O or EB+P (see Chapter 2, Graham & Pfaus, 2013).

Perhaps the most studied neurotransmitter in the mPOA is also the most complex. DA in the mPOA plays a prominent role in the display of precopulatory behaviours, and this role is dependent upon the hormonal profile of the female. When females are injected with only EB, an increase in precopulatory behaviours is seen following an infusion of a DA D2R or non-selective DAR agonist to the mPOA, but a decrease is observed when a DA D1R agonist is given (Graham & Pfaus, 2010). In females injected with both EB+P, increases in precopulatory behaviours are seen following an mPOA infusion of a DA D2R antagonist, but a decrease is found when a DA D1R or non-selective antagonist is given. Collectively this data implies that EB-alone females have a DA D1R/D2R ratio in favour of DA D2R, with DA D2R activity being dominant in promoting sexual motivation. The administration of P in addition to EB shift the DA D1R/D2R ratio in favour of DA D1R, indicating that in these females DA D1R activity facilitates sexual motivation. This demonstrates that the role of DAR subtypes can be altered by ovarian hormones, and specifically P. DA is released in the mPOA of rats treated with EB+P, but not

rats treated with only EB (Matuszewich et al., 2000), and extracellular DOPAC increases in the mPOA around the time sexual behaviour becomes activated (Luine, 1993). This helps to explain the facilitatory role of ascorbic acid in the mPOA on precopulatory behaviours (see Chapter 2, Graham & Pfaus, 2013), as ascorbic acid has been shown to increase extracellular DA concentration, and greater extracellular diffusion through the prevention of DA oxidation (Morales, Fuentes, Ballaz, Obeso, & Rodriguez, 2012). Similarly, the effect of bremelanotide can be explained as systemic administration stimulates DA release in the mPOA of females treated with EB+P, but not in the VMH or NAc. Furthermore, the effects of bremelanotide can be blocked by administration of a DA D1R antagonist to the mPOA (Pfaus et al., 2007). This suggests in fully hormonally-primed females that DA terminals in the mPOA contain MC4 receptors. Activation of these receptors drive the release of DA which facilitates precopulatory behaviours by acting on DA D1R.

The Control of the mPOA on Timing Mechanisms

The ratio of DAR in the mPOA could act as a control mechanism that modulates the timing and display of the different components of sexual behaviour. The most immediate and short-term of the effects may act to trigger the onset and offset of different sexual behaviour components. Stimulation of DA D1R, perhaps through phasic DA release, will result in a promotion of precopulatory behaviours, thus signalling to the male the female wishes to copulate. This facilitation of precopulatory behaviours could be accompanied by communication with areas such as the VMH and the ArcN to inhibit lordosis, as both these behaviours cannot occur at the same time. When enough DA is released, the summation of DA might spillover past DA D1R and act to stimulate DA D2R. Simultaneously, since the phasic release is done in a brief, pulsatile manner, and through reuptake DA is removed quickly from the synaptic cleft (Grace, 1991; Grace, 2002), stimulation of the DA D1R will cease. This coinciding DA D2R activation and DA D1R inactivation may result in a "shutting off" of the precopulatory behaviours, while also signalling other areas to disinhibit the tonic inhibition of lordosis via the mPOA connections to the VMH, ArcN, PAG, and MRF (see Figure 2 and Figure 3). Interestingly, this mechanism is reminiscent to the "flip-flop" switch originally proposed by Saper and colleagues (2001) for the control of sleep by hypothalamic areas such as the ventrolateral preoptic nucleus, with GABA having a major role within this switch (Saper, Chou,

& Scammell, 2001). These two similar proposals suggest an overarching mechanism for hypothalamic control of various motivational systems that warrants further examination.

Modification of the receptor levels by ovarian hormones may be an important mechanism for the control of sexual motivation. Administration of only EB may result in either more DA D2R or less competition from endogenous DA, and under these conditions the female may be driven to engage in sexual activity. Even though it is strictly an artificial phenomenon produced in the laboratory, this could be a glimpse of a mechanism that could be important in the control of the longer-term timing aspects of female sexual behaviour. Examples of this could include estrus termination and/or menopause onset.

An additional role of EB can have a more long-term role in the timing of female sexual behaviour. EB administration (with or without P) decreases the proportion of GABAergic neurons containing DA D1R projecting from the mPOA to the VTA (see Chapter 4). Since GABA receptors in the VTA inhibit DAergic activity (Johnson & North, 1992), this could increase the sensitivity to sexual reward for the female. Acting in this way, it could be that this mPOA to VTA projection is an important mechanism primed via an increase in estradiol to help prepare the female to engage in sexual behaviour by increasing her responsiveness to incentives that are associated with sexual reward (see Figure 3). This modification may or may not also have a role in reducing GABA-mediated inhibition, which would allow for the VTA to facilitate lordosis (Frye, 2001; Frye, 2011). Whichever means utilized, this could be imperative in laying the foundation for the female to want to engage in sexual behaviour, and thus can be thought of as important for the initiation of sexual behaviour.

Estrus termination is the endocrine transition to pregnancy (or pseudopregnancy), resulting in an inhibitory state that is part of the refractory period of the female (Erskine, 1985; Pfaus, Smith, & Coopersmith, 1999; Pfaus, Smith, Byrne, & Stephens, 2000). In a laboratory setting, it has shown to be induced by mating stimulation or VCS (Lodder & Zeilmaker, 1976), and it is characterized by a decrease in precopulatory behaviours accompanied by an increase in aggressive rejection responses (Pfaus et al., 1999; Pfaus et al., 2000). In a natural setting this typically results in females removing themselves from the mating environment by going into a different burrow. Beside genital stimulation, it can occur due to a natural decline in hormone actions, e.g., in the absence of estradiol-bound ERs (Blaustein, Dudley, Gray, Roy, & Wade, 1979), and/or the decline in occupied PRs (Blaustein & Olster, 1989), or due to the

downregulation of PRs (and PR-B specifically; Gomez-Camarillo, Beyer, Lucio, Garcia-Juarez, Gonzalez-Arenas, Camacho-Arroyo, Komisaruk, & Gonzalez-Flores, 2011). The onset of estrus termination may be due to the downregulation of ER and PR by P in tandem with the decrease in E2 levels resulting in a loss of induced PRs. It should be added however, that this onset is likely facilitated by VCS-induced activation of inhibitory glutamate action in the VMH (Georgescu et al., 2009; Jones et al., 2017).

The very beginning of estrus termination could also be initiated by a change in DAR ratio in the mPOA, resulting in the first behavioural expression that is a decrease in sexual desire. Expanding on this idea, the DAR ratio in the mPOA could be fundamental in controlling precopulatory behaviour in the female, and the hormone levels of EB, and more importantly P, can produce ratios that facilitate these behaviours. When females are fully hormonal-primed, the DA D1R/D2R ratio becomes biased towards DA D1R, and these females show increased precopulatory behaviours relative to females primed with EB+O or O+O. Females primed under these conditions do not have more DA D1R relative to DA D2R, and thus rarely exhibit these behaviours. As ovarian hormone levels start to decrease, perhaps the ratio of DARs are returned to a low DA D1R/D2R state and phasic DA release no longer results in enough activation of DA D1R to promote precopulatory behaviours.

Lordosis can be stimulated for a longer period of time, however, perhaps due to tonic DA release activating DA D2R, continuing to disinhibit the tonic inhibition of lordosis through connections with areas such as the VMH, ArcN, PAG, and MRF. Lordosis also eventually ends, and this appears to be due to the activation of glutamate neurons in the VMHvl (Georgescu, Sabongui, Del Corpo, Marson, & Pfaus, 2009), and these glutamate neurons also contain GABA-A receptors (Georgescu et al., 2014). Based on the results of Chapter 4 of the current thesis, EB increases the proportion of mPOA connections to the VMH that are GABAergic, and that have DA D2R located on them. This occurs regardless of if P was administered. This change in pathway could be the source of GABAergic input to glutamate neurons in the VMH that results in the second part of estrus termination. Specifically, its role may be to turn off lordosis completely and start the female refractory period. Furthermore, the results of Chapter 4 also show that under EB+P conditions there is an increase of DA D1R on GABAergic neurons projecting from the mPOA to the VMH. This may act to further prolong sexual receptivity, delaying estrus termination due to the increase in proportion, relative to other hormonal

conditions. A similar system could be in place regarding menopause onset, and this mechanism could help to explain the libido decrease that can be a symptom of that condition, in response to decreased levels of fluctuating hormones that accompany the female at that time.

The mPOA appears to be at the centre of the three major systems that are responsible for collaborating and producing successful female sexual behaviour focused on a specific, appropriate male. This role of DA and its receptors within this area further seems to be responsible for both the onset and the offset of these systems. This could occur in the short term within intermediate copulatory bouts. It could also be important in the longer aspect of timing by first initiating, and later shutting down a completed session. This would likely transpire in correspondence with hormonal and sensory influences. Further research should focus on manipulating DA and DAR to determine if changes in sexual behaviour timing mechanisms are also altered.

CHAPTER 6: GENERAL DISCUSSION

This thesis examined DA-related mechanisms in the mPOA important for the display of female sexual behaviour in the rat, and how hormone levels may alter these mechanisms. These potentially exist so that the behaviours are exhibited at appropriate times throughout the reproductive cycle. Previous work from my Master's thesis revealed that administration of DA DAR selective agonists and antagonists to the mPOA differentially regulate precopulatory behaviour in the female rat depending on her hormonal profile (Graham & Pfaus, 2010; Graham & Pfaus, 2012). Females that had been primed with EB alone seemed to be under a DA D2R-mediated system, such that DA D2R stimulation increased precopulatory behaviour. Females primed fully with EB+P switched to a DA D1R-mediated system, as precopulatory behaviours in these animals were increased by DA D1R stimulation. Thus, the purpose of the present thesis was to investigate further this DAR switch and elucidate its action as a driver of brain mechanisms responsible for both appetitive and consummatory female sexual behaviours.

The first experiment (Chapter 2) served to extend our previous behavioural findings by examining whether endogenous DA would show similar results to exogenous administration of agents acting postsynaptically on DAR. Findings replicated the work from my Master's thesis in that administration of AA increased solicitational behaviour while having no effect on lordosis. This solidified our understanding that DA in the mPOA is critical to the control of solicitational behaviour, contributing to how sexual behaviours in the female rat are timed.

The next set of experiments (Chapter 3) was designed to examine the presence of a DAR switch under different hormonal conditions, bringing several techniques to bear on the question. Immunohistochemistry was utilized as a beginning step to indirectly analyze whether cells stained for each type of DA subclass, DA D1R and DA D2R, differed in number in the mPOA under three hormone profiles: oil-alone control (O+O), partial hormone-priming with estradiol benzoate alone (EB+O), and full hormone-priming with the addition of progesterone (EB+P). It was found that females primed with EB+O had a lower number of DA D1R-stained cells than the other two groups. A linear trend was also found, indicating that EB+P primed females had a lower number of DA D2R-stained cells. The combination of these results produced a significant difference between the two experimental groups in regards to DAR ratio, with EB+P females having a significantly greater log-transformed DA D1R/D2R ratio compared to the EB+O group.

This provided the first evidence that a physiological change to DAR in the mPOA was occurring due to hormonal manipulation.

The immunohistochemistry study was followed by further analysis of the types of changes that might occur under the different hormonal conditions. To accomplish this, Western blotting was performed in the mPOA of females given the same three hormonal priming regimens. This allowed for the quantification and analysis of protein levels for the two DAR subtypes. Blots were done for the mPOA and two control areas, the PLC and the CP, which were expected to act as negative and positive control areas, respectively. The results indicated that females primed with EB+O showed lower DA D1R and higher DA D2R protein levels when compared to the other two hormone groups. For females primed with EB+P, protein levels return to that of control levels, and in fact surpass the O+O levels. This produced DAR ratios that were similar to those found with immunohistochemistry, indicating that EB+P primed females having a significantly higher log-transformed DA D1R/D2R ratio compared to the EB+O group. This study therefore provided additional evidence that hormonal manipulations caused physiological changes to DAR in the mPOA, specifically altering DAR protein levels complementary to the behavioural effects previously observed (Graham & Pfaus, 2010; Graham & Pfaus, 2012). Surprisingly, protein level changes were not observed in the CP, an area chosen under the expectation it would act as a positive control, due to various findings that DA is altered under chronic hormone administration in the CP (e.g., Hruska et al., 1980; Hruska, 1986; Hruska & Nowak,1988; Fernandez-Ruiz et al., 1989; Levesque & Di Paolo, 1989). Also surprising was that some protein level changes were found in the PLC, an area chosen as a negative control due to a lack of published studies finding hormonal alterations of DA in this area. Contrary to our expectations of no differences, findings similar to the mPOA were observed. Specifically, decreased DA D1R and increased DA D2R protein levels found in EB+O primed females in comparison to those primed with both EB+P. This resulted in a significant decrease in log-transformed DA D1R/D2R ratio for the EB+O group compared to the other two groups.

To supplement the Western blot findings, autoradiography was employed to determine if hormonal administration also alters the functional binding profiles of DARs in the mPOA. Females from the same three hormonal groups were compared (O+O, EB+O, EB+P), and functional binding was analyzed in the same three areas (mPOA, PLC, CP). None of the findings were statistically significant, possibly due to power and/or floor effects in terms of DAR

functional binding levels. However, effect sizes indicate that functional binding levels of DA D2R may be increased in the more central portion of the mPOA in females primed with EB+P. This finding was accompanied by a much lower log-transformed DA D1R/D2R ratio. Although this result is the opposite of what we found for the immunohistochemistry and Western blotting studies, it likely reflects less DA binding to DA D2R in EB+P conditions, an interpretation consistent with all three studies. This is reminiscent of the inverse algorithm employed for radiolabelled spiperone in positron emission tomography studies of DA terminal regions such as the NAc. In these types of studies, a decrease in label indicates more endogenous DA released because DA binds competitively with the antagonist (Laruelle et al., 1997a; Laruelle, Iyer, al-Tikriti, Zea-Ponce, Malison, Zoghbi, Baldwin, Kung, Charney, Hoffer, Innis, & Bradberry, 1997b). Finally, no functional binding differences were found in the PLC or CP.

Taken together, the studies of Chapter 3 demonstrate that administration of exogenous steroid hormones to OVX females can have physiological changes that presumably are responsible for behavioural differences observed previously (Graham & Pfaus, 2010; Graham & Pfaus, 2012). These changes were seen in potentially three different aspects: indirect DAR number by counting cells stained for DAR using immunohistochemistry; DAR protein level using Western blotting; and DAR functional binding using autoradiography. These techniques support the behavioural findings that females partially primed with EB-alone have a DA D2R-mediated system responsible for the display of precopulatory sexual behaviours. The addition of P, resulting in fully-primed females, switches this system to DA D1R-mediation.

The studies of Chapter 4 were performed to understand the neurochemical identity of cells in the mPOA that show the DAR alterations induced by priming with EB and EB+P. This was done to help understand the anatomical pathways responsible for communicating the DAR changes seen in Chapter 3. To achieve this, the retrograde tracer Fluoro-Gold was infused into two areas known to be important in the control of female sexual behaviour, and specifically lordosis: the VMH and the VTA. Fluorescence immunohistochemistry was also employed to stain for the presence of glutamate or GABA, indicating neuronal type. Thus, examination of the mPOA for evidence of the FG tracer in conjunction with glutamate or GABA staining would allow us to determine if projections from the mPOA to the VMH or VTA consisted of these types of neurons. Finally, fluorescent staining was also performed for the two DAR subtypes, providing demonstration of evidence that these connections may be under DAergic control, and

if so which receptor subtype is activated when sending signals. These stainings were done on the same three hormonal groups compared in Chapter 3, specifically O+O, EB+O, and EB+P.

Exploration of the mPOA projections to the VMH indicated that in females fully hormonally-primed with EB+P these connections are more often composed of glutamate neurons and more likely to have DA D2R on them. When given EB-alone however, a higher proportion of neurons projecting there were GABAergic, regardless of subsequent P administration. The proportion of DA D2R located on these neurons was also increased under this hormonal milieu. However, when P was administered after EB, an increase of DA D1R was seen instead. Projections from the mPOA to the VTA that are GABAergic and have DA D1R on them were decreased when females are given EB, with or without P. Finally, the proportion of DA D2R-stained cells that are also glutamatergic that do not project to the VMH or the VTA was increased by EB+P administration. This indicates that at least one more area may be important in terms of this specific mPOA DAR-mediated female sexual behaviour neural network.

Overall, the primary findings of this thesis help establish a mechanism that may be responsible for the organization of an imperative system necessary for reproductive success, as discussed in Chapter 5. The observation of physiological alterations in neurotransmitters, both in functional binding, protein levels, and indirectly via their occupation on counted neurons, can help explain behavioural effects previously seen. These findings were extended to include neuronal projections to important areas for female sexual behaviour. This provides evidence of larger-scale systematic changes that coordinate disparate sensory inputs (e.g., olfactory, clitoral, vaginocervical, flank) and the behavioural response to them during copulation (see Figure 1).

The Ratio of Dopamine Receptors on Behaviour

The ratio of DARs has been hypothesized to underlie a wide variety of mental states and behaviours, including schizophrenia (Hess et al., 1987; Winterer & Weinberger, 2004), aging (Seeman et al., 1987), circling behaviour (Glick et al., 1988; Roy et al., 1990), dyskinesia (Gagnon et al., 1993), pain (Hagelberg et al., 2003a; Hagelberg et al., 2003b), and locomotion (Spencer et al., 2012). It is speculated to be involved in the control of male sexual behaviour in a variety of animals (Kleitz et al., 2009). The two receptor subtypes have been shown to be synergistic in many psychomotor activities such as Huntington's disease, Parkinson's, and schizophrenia (Seeman, Niznik, Guan, Booth, & Ulpian, 1989), and to have opposing roles in

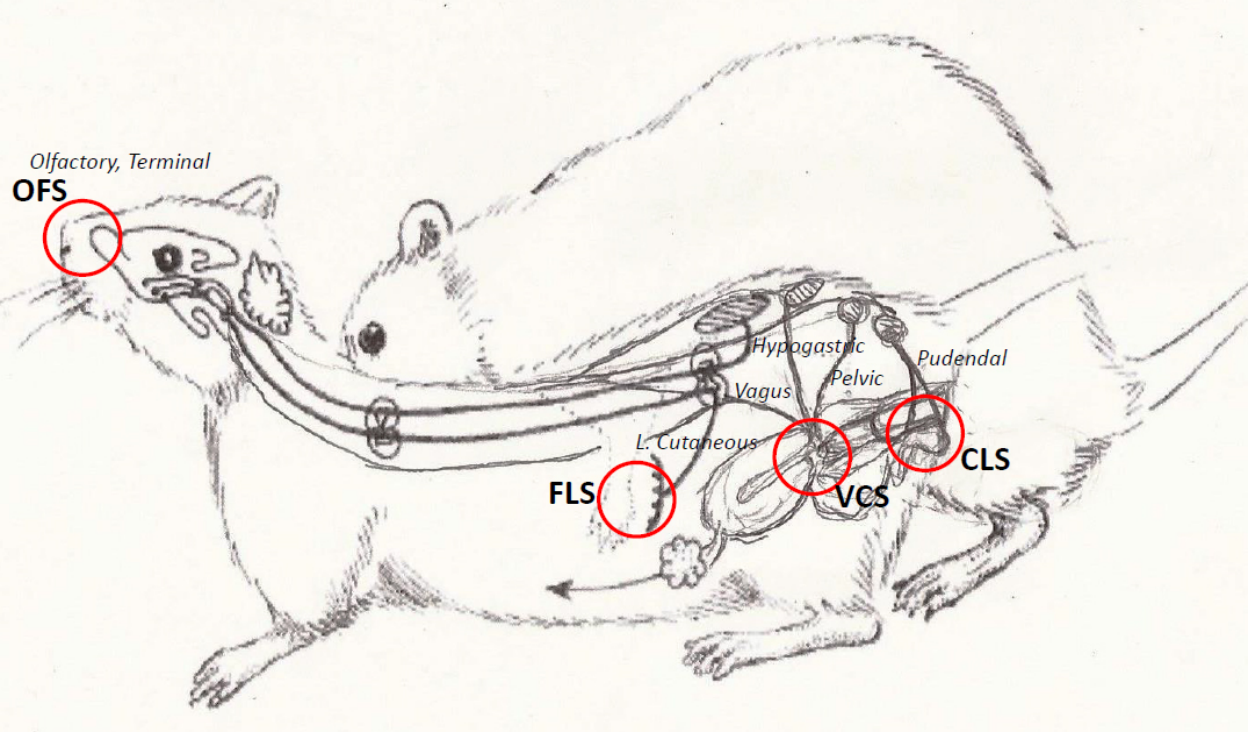

Figure 1. Diagram indicating the different aspects of sensory inputs received by the female rat during copulation. (Adapted from Pfaus, Jones, Flanagan-Cato, & Blaustein, 2015. Reprinted with permission from Elsevier).

male sexual behaviour (Hull et al., 1989; Markowski et al., 1994; Moses et al., 1995) and in the formation of sexual bonds (Aragona, Liu, Yu, Curtis, Detwiler, Insel, & Wang, 2006). These interactions between receptor subtypes could occur naturally in neurons that have both receptors on their surface through a shuttling of the beta-gamma subunits of the G proteins associated with each respective DAR (Seeman et al., 1989). Specifically, the beta-gamma subunit is identical for both the DA D1R-associated Gs protein and the DA D2R-associated Gi protein. This subunit "shuttles" between the alpha subunits for the Gs and Gi proteins. This has been shown to result in an interaction between DA D1R and DA D2R (Seeman, 2000). This shuttling may help the DA D1R keep the DA D2R in its low-affinity state, and if there is a reduction in the DA D1R, a resulting occurrence could be an increase in the DA D2R retaining its high-affinity state, which serves as its functional state (Seeman, 2000). Therefore, a decrease in the production of DA D1R may result in an increase in functional DA D2R, which could impact a wide variety of behaviours, including female sexual behaviour under different hormonal conditions. Alternatively, interactions between the two DAR subtypes may occur through a neuron-neuron interaction. This could be the case if different neurons have different receptor subtypes located on them. This could also occur through an intracellular interaction in the same cell if both DA D1R and DA D2R are co-localized. Evidence exists of both types of these interactions (for a review, see Seeman, 2000). Further research should be performed to help determine which possibility, or combination of possibilities, may be driving the DAR ratio differences in response to ovarian hormones.

Effects of Ovarian Steroids on Dopamine

Estrogen and Dopamine

The exact mechanism(s) by which ovarian steroid hormones may interact with DA has yet to be elucidated, but various effects have been discovered. Due to the delay in the effects seen in the experiments of Chapters 3 and 4 (48 and 4 hours after estrogen and progesterone administration, respectively), classical genome-activating ERs seem to be most likely responsible. Furthermore, these changes could be in concordance with the later, more rapid onset changes seen in the striatum and NAc that potentially control pacing behaviour (Becker, 1999). It could be this combination of slow-acting and rapid effects that drive the sexual desire of female rats. In accordance with this hypothesis, two types of nuclear estrogen receptors (ERα and ERβ) have been found to exist in many brain areas, including the mPOA (Shughrue et al.,

1997). The striatum however, has minimal, if any, ERα (Pfaff & Keiner, 1973), and no ERβ has been found there (Shughrue et al., 1997). The rapid effects seen in the striatum may instead be caused by a recently identified non-classical G protein-coupled estrogen receptor 1 (GPER1) that functions as a membrane ER (Prossnitz & Barton, 2009). These receptors have been found in the striatum, including the CP, in moderate amounts (Hazell et al., 2009; Prossnitz & Barton, 2009), and in intense amounts in the mPOA (Hazell et al., 2009). Although no research has examined the effects of GPER1 on DA systems in the rat brain, GPER1 is capable of both transcriptional and rapid actions in response to estrogen (Prossnitz & Barton, 2009), and the form of estradiol used in the present study (17β-estradiol) does bind to it (Thomas, Pang, Filardo, & Dong, 2005). This allows for the possibility that instead of the DAergic effects seen in the mPOA being due to classical ERs, perhaps estrogen acting on GPER1 is part of the driving force behind DA and its role on sexual desire in the female rat.

Finally, it is possible that E2 administration is altering DA levels through changes in tyrosine hydroxylase (TH), which catalyzes the conversion of amino acids L-tyrosine to L-dihydroxyphenylalanine (L-DOPA). Although TH acts as the rate-limiting enzyme in the biosynthesis of all catecholamines, and thus is not specific to DA, it has been found that long-term E2 administration can differentially affect TH levels in different areas of the rat brain. In the mPOA, this long-term treatment increases the number of TH-immunoreactive neurons, while decreasing these levels in the nearby periventricular preoptic nucleus (PPN) (Yuri & Kawata, 1994). A single administration of EB has also been shown to decrease TH-immunoreactive neurons in the substantia nigra compacta, and two areas of the VTA (Zsarnovszky, Scalise, Horvath, & Naftolin, 2000). Further studying of the impact of acute administration of EB on TH levels in the mPOA is warranted.

Progesterone and Dopamine

Similar possibilities exist for how P may interact with DA in the mPOA, as evidence demonstrates different actions for P overall in different regions important for female sexual behaviour. In the VMH, the binding of P to traditional intracellular progesterone receptors (PR) facilitates lordosis (for a review, see Frye, 2001). In the VTA however, the facilitation of lordosis is instead independent of PRs, through rapid actions of P at non-genomic neuronal membranes (for a review, see Frye & Walf, 2008). Evidence also shows that P interacts with DA in brain areas important for female sexual behaviour, for example increasing DA levels in the

VTA (Frye et al., 2000) and DA D1R density in the striatum (Dluzen & Ramirez, 1989; Levesque & Di Paolo, 1990). In the CP, P decreases DA D2R four hours after independent administration, an effect not seen when EB is given prior. However, there was an increase in DA D2R levels 24 hours after EB+P treatment (Fernandez-Ruiz et al., 1989).

Progesterone likely has a more direct interaction with DA in terms of female sexual behaviour, as it plays a role in DAR-related second messenger actions. For example, Mani and colleagues have demonstrated that the facilitation of female sexual behaviour in rodents is due to the existence of cross-talk between signalling pathways activated by P and the DA D1R agonist SKF 38393 (Mani et al., 1994; Mani et al., 1996). Later it was discovered that an attenuation of sexual receptivity is seen in rats following infusions of anti-sense oligonucleotides that block DARPP-32, a DA- and adenosine 39,59-monophosphate (cAMP)-regulated phosphoprotein, indicating that DARPP-32 activation is necessary for sexual receptivity induced by both PR regulation and DA facilitation (Mani et al., 2000). Furthermore, although both mechanisms result in the activation of cAMP-mediated protein kinase A (PKA), which ultimately leads to the phosphorylation of DARPP-32, the interdependent signal transduction pathways initiated by both P and SKF 38393 are surprisingly independent of each other, indicating that these two mechanisms activate distinct upstream signalling kinase pathways (Mani et al., 2000). Progesterone has also been shown to increase cAMP levels in the mPOA and other regions four hours following injection (Collado et al., 1985), and both systemic injections and direct infusions of cAMP into the brain stimulates lordosis in EB-primed rats (Beyer et al., 1981). Furthermore, reducing cAMP degradation via administration of phosphodiesterase inhibitors also enhances lordosis in rats given low doses of P (Beyer & Canchola, 1981). Several studies by Frye and colleagues have demonstrated that the non-genomic actions of P in the VTA on lordosis also require a role for DA and its downstream components (for a review, see Frye & Walf, 2008).

In regards to P having an effect on TH levels, administration of P has been showed to decrease TH mRNA in the ArcN and PPN (Morrell, Rosenthal, McCabe, Harrington, Chikaraishi, & Pfaff, 1989; Arbogast & Voogt, 1994), although double-labelling studies have found colocalization of PR and TH only in the ArcN of guinea pigs, but not the PPN (Blaustein & Turcotte, 1989a). To the best of our knowledge, no research to date has looked at the effect of P on TH in the mPOA, and a lack of colocalization of PR and TH has been found in the preoptic area of guinea pigs (Lemoine, Leroy, & Warembourg, 2005).

Testosterone and Dopamine

Dopamine levels may also play a role in female sexual behaviour by acting as an intermediary for testosterone. Testosterone is well-known to increase sexual desire in women (e.g., Burger, Hailes, Menelaus, Nelson, Hudson, & Balazs, 1984; Burger, Hailes, Nelson, & Menelaus, 1987; Sherwin, Gelfand, & Brender, 1985; Shifren, Braunstein, Simon, Casson, Buster, Redmond, Burki, Ginsburg, Rosen, Leiblum, Caramelli, & Mazer, 2000) and female rats (Sanchez et al., 2010; Jones, Ismail, King, & Pfaus, 2012) and may have its effects using DA as a substrate. Testosterone fluctuation is similar in rats and women (Belanger et al., 1981; Hall Moran, Leathard, & Coley, 2001; McCarthy & Becker, 2002; Pfaus et al., 2015), peaking in rats in the morning of proestrus after a rise during the afternoon of diestrus (Belanger et al., 1981). In male rats, T is required for the release of DA in the mPOA, which has been shown to be necessary for male rat copulation, genital reflexes, and sexual motivation (Hull et al., 1999). Testosterone increases neuronal nitric oxide synthase (NOS; reviewed in Hull & Dominguez, 2006; Hull, 2011), an enzyme that produces the gaseous messenger molecule nitric oxide, which in turn increases DA release (Hanbauer, Wink, Osawa, Edelman, & Gally, 1992; Zhu & Luo, 1992; Lorrain & Hull, 1993), thus facilitating male copulation. Alternatively, T may have its effects via E2 after being aromatized by the enzyme aromatase (Bancroft, 2002), in which case it would act on ER, which may or may not involve DA. The effect of T on NOS may also be through aromatization, as estradiol upregulates neuronal NOS in the mPOA (Putnam, Sato, Riolo, & Hull, 2005; Sato, Braham, Putnam, & Hull, 2005).

Testosterone has shown to have effects on the gene expression of DA and DAR in male adolescent rats, and this occurs via androgen receptors (AR). Specifically, T-induced activation of AR attenuates DA turnover in the striatum, and modulates mRNA levels of multiple DAR, including increasing DA D1R and both the long and short form of DA D2R in the substantia nigra (Purves-Tyson, Owens, Double, Desai, Handelsman, & Weickert, 2014). In women, however, striatal DA has been found to increase following exogenous T, as evidenced by an elevated BOLD response during functional magnetic resonance imaging (Hermans, Bos, Ossewaarde, Ramsey, Fernandez, & van Honk, 2010). This effect was seen in anticipation of potential rewards, and was in fact stronger for those women with lower intrinsic appetitive motivation, as women with high intrinsic appetitive motivation did not show a further increase in striatal DA following T administration (Hermans et al., 2010).

The 5α-reduced metabolite of T, DHT, has been shown to decrease sexual receptivity in several rodent species. One possible way it does this is to inhibit neural binding of administered estradiol by competing with ER (for a review, see Frye, 2001). Alternatively, it may act through its metabolism to 3α-androstanediol, which may act via a non-genomic mechanism due to its low affinity for AR (Roselli, Horton, & Resko, 1987; Frye, 2001). Serum levels of 3α-androstanediol are at their highest during the evening of proestrus, coinciding with when female rats are typically most sexually receptive. Serum levels then decline in estrus, when sexual behaviour is diminished (for a review, see Frye, 2001).

Androgen receptor expression may be affected by progestins and/or their receptors. Male progesterone receptor knockout (PRKO) mice have been found to have an increase in AR expression in multiple brain areas, including the mPOA (Schneider, Burgess, Sleiter, DonCarlos, Lydon, O'Malley, & Levine, 2005), and an increase in AR expression has been shown to be related to increases in male sexual behaviour (Meisel & Sachs, 1994). In female macaques and humans, endometrium AR expression increases following an acute injection of the synthetic PR antagonist RU-486 (Slayden, Nayak, Burton, Chwalisz, Cameron, Critchley, Baird, & Brenner, 2001; Narvekar, Cameron, Critchley, Lin, Cheng, & Baird, 2004). It has also been shown that RU-486 increases AR activity (Kemppainen, Lane, Sar, & Wilson, 1992). It has been suggested (e.g., Forbes-Lorman, Auger, & Auger, 2014) that the influence of ARs may instead occur indirectly through mediation of estrogen, as progestins have been shown to down-regulate ER in vitro (Read, Green, & Katzenellenbogen, 1989; Alexander, Shine, & Sutherland, 1990; DonCarlos, McAbee, Ramer-Quinn, & Stancik, 1995). Taken together, it is possible that the hormonal administrations in the studies of the current thesis result in differing levels of AR expression, which T acts on to interact with DA and/or DAR.

DA and Sexual Desire Disorders

The interaction of T and DA in female sexual behaviour has a major implication for sexual desire disorders in women both pre- and post-menopausal. Hypoactive sexual desire disorder (HSDD) is affected by menopausal status, with the highest prevalence in younger surgically-menopausal women (16-26%). Slightly lower, but still problematic are premenopausal (7-14%) and naturally-menopausal women (6.6-9%; Dennerstein, Koochaki, Barton, & Graziottin, 2006; Leiblum, Symonds, Moore, Soni, Steinberg, & Sisson, 2006; West, D'Aloisio, Agans, Kalsbeek, Borisov, & Thorp, 2008). About 300μg of T is produced daily by

premenopausal women (Southren, Gordon, & Tochimoto, 1968), which is reduced by approximately 50% through natural menopause (Judd, 1976) or by 80% through bilateral oophorectomy (Hughes, Wall, & Creasman, 1991). Daily apomorphine, a nonspecific DA agonist, may be effective in treating some premenopausal women with normal T levels for HSDD (Caruso, Agnello, Intelisano, Farina, Di Mari, & Cianci, 2004) and orgasmic dysfunction (Bechara, Bertolino, Casabe, & Fredotovich, 2004). In postmenopausal women with sexual dysfunction, Goldstein (2007) proposes a clinical paradigm for treatment incorporating in Phase 3 DA agonists in combination with steroid hormones (EB, P, and/or T).

Taken together, the findings of this thesis have application in the treatment of female sexual interest/arousal disorder (FSIAD), characterized by a severe reduction, or lack of, sexual arousal and/or interest. In the most recent version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), FSIAD has become a combination of two previously separate disorders: HSDD and female sexual arousal disorder (FSAD) (American Psychological Association, 2013; IsHak & Tobia, 2013). However, there is controversy concerning this new nomenclature, and these two disorders will remain separate in the ICD-11 as recommended by the International Society for Sexual Medicine and the International Society for the Study of Women's Sexual Health (Goldstein, Kim, Clayton, DeRogatis, Giraldi, Parish, Pfaus, Simon, Kingsberg, Meston, Stahl, Wallen, & Worsley, 2017). Currently there are several pharmacological treatments for HSDD either approved or in late Phase III clinical trials that can help shed light on the mechanisms explored in this thesis.

Flibanserin

Flibanserin has recently been approved for treatment of HSDD in the United States, becoming the first such treatment in the world. It is a mixed 5-HT1A agonist and 5-HT2A antagonist which has been demonstrated to improve sexual desire and reduce sexual distress in women who suffer from HSDD (DeRogatis, Komer, Katz, Moreau, Kimura, Garcia, Wunderlich, & Pyke, 2012; Thorp, Simon, Dattani, Taylor, Kimura, Garcia, Lesko, & Pyke, 2012; Katz, DeRogatis, Ackerman, Hedges, Lesko, Garcia, & Sand, 2013). Flibanserin also increases solicitations, hops and darts, and other precopulatory behaviours in female rats primed with a low dose of EB, and decreases their display of rejection responses (Gelez et al., 2010; Gelez, Greggain-Mohr, Pfaus, Allers, & Giuliano, 2013). Activation of certain 5-HT receptors inhibits DA release, including postsynaptic 5-HT1A and 5-HT2A (Di Matteo, Di Giovanni, Pierucci, &

Esposito, 2008). Although the exact mechanism(s) underlying the effects of flibanserin are not fully understood (Stahl, Sommer, & Allers, 2011), female rats show an increase in DA levels in the mPOA following acute administration (Allers, Dremencov, Ceci, Flik, Ferger, Cremers, Ittrich, & Sommer, 2010), and increased Fos activation in the mPOA following chronic administration of the only dose that increases precopulatory behaviours (Gelez et al., 2013). This corresponds nicely with the findings of the present thesis, in that the additional behavioural effects observed can be explained by neurophysiological changes found by the studies conducted here within. It appears that the effects of flibanserin in augmenting sexual desire in women and rats may be due to changes in the mPOA, specifically an increase in neural activation of that area and an increase in DA levels which would interact with areas as previously described in the aforementioned model of Chapter 5.

Bremelanotide

Another potentially promising avenue of treatment for women with HSDD is the MC4 receptor agonist bremelanotide. Although its use as an intranasal spray was initially halted over potentially worrisome side effects causing increased blood pressure, trials have now been resumed using it as a subcutaneous self-injection, with preliminary results indicating an increase in satisfying sexual events following administration (Kingsberg et al., 2015). As previously discussed, bremelanotide acts as a MC agonist through MC3 and MC4 receptors, the latter of which are found in the mPOA (Gelez et al., 2010), acting on DA D1R to cause DA release in this area (Pfaus et al., 2007). Thus, the ability for bremelanotide to increase female sexual desire appears to act through the DAR mechanism in the mPOA via MCs, which would set off a cascade of downstream effects as seen in the model described in Chapter 5.

PDE-5 Inhibitors

Following the success of phosphodiesterase type 5 (PDE-5) inhibitors such as Viagra (sildenafil), Cialis (tadalafil), and Levitra (vardenafil) in reducing male erectile dysfunction, the idea of using them to treat female sexual disorders was an obvious next step. In females, genital vasodilation and engorgement results from the relaxation of smooth muscle in both the clitoris and vagina, a process caused by downstream effects of nitric oxide (NO) activating guanylyl cyclase to form cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (Gragasin, Michelakis, Hogan, Moudgil, Hashimoto, Wu, Bonnet, Haromy, & Archer, 2004; Ückert, Kuczyk, & Oelke, 2013). PDE-5s typically break down cGMP, and thus PDE-5

inhibitors block this breakdown, resulting in cGMP having a longer effect on the smooth muscles. PDE-5s are however found in much smaller quantities in the female genitalia relative to the penis (Oelke, Hedlund, Albrecht, Ellinghaus, Stief, Jonas, Andersson, & Ückert, 2006; Ückert, Ellinghaus, Albrecht, Jonas, & Oelke, 2007).

The findings of studies using PDE-5 inhibitors (most commonly sildenafil) in the treatment of FSIAD is mixed, and the common consensus appears to be that PDE-5 inhibitors may have some use in treating disorders due to genital dysfunction in women, but might not be beneficial to women suffering from sexual desire disorders (Schoen & Bachmann, 2009; Gao, Yang, Yu, & Cui, 2015). This is unsurprising since PDE-5 inhibitors have no effect on sexual desire in men either (Goldstein, Lue, Padma-Nathan, Rosen, Steers, & Wicker, 1998; Jones, Klimberg, McMurray, Padula, Tseng, & Stecher, 2008). Data from the animal literature back this up, as vardenafil did not have an effect on female sexual behaviour in rats (Snoeren, Bovens, Refsgaard, Westphal, Waldinger, Olivier, & Oosting, 2011), an effect that is somewhat unexpected given that sildenafil enhances DAergic transmission in the mPOA of male rats (Kyratsas, Dalla, Anderzhanova, Polissidis, Kokras, Konstantinides, & Papadopoulou-Daifoti, 2013). It has been demonstrated in males that DA release in the mPOA is modulated by NO (Dominguez, Muschamp, Schmich, & Hull, 2004), and as mentioned NO results in the formation of cGMP, a target of PDE-5s.

A new strategy to combat female sexual desire disorders is to combine a PDE-5 inhibitor (specifically sildenafil) with T, marketed under the name Lybrido. The rationale behind this combination is that the PDE-5 inhibitor enhances genital sexual response in combination with T increasing the response of the brain to sexual cues (Poels, Bloemers, van Rooij, Koppeschaar, Olivier, & Tuiten, 2014), and thus it is probably intended for women with a decreased sensitivity to sexual stimuli resulting in HSDD (Kingsberg et al., 2015). Preliminary evidence shows that Lybrido is effective in treating women with HSDD and FSAD (Poels, Bloemers, van Rooij, Goldstein, Gerritsen, van Ham, van Mameren, Chivers, Everaerd, Koppeschaar, Olivier, & Tuiten, 2013; van Rooij, Poels, Worst, Bloemers, Koppeschaar, Goldstein, Olivier, & Tuiten, 2015), as is T in combination with vardenafil (van der Made, Bloemers, Yassem, Kleiverda, Everaerd, van Ham, Olivier, Koppeschaar, & Tuiten, 2009). Animal studies reinforce the human data, as co-treatment of T and vardenafil increases both precopulatory and consummatory behaviour in female rats, however only when they are hormonally-primed with EB-alone and not

when administered EB+P (Snoeren et al., 2011). Although lordosis quotients were affected in females given either 5mg or 2mg of EB, precopulatory behaviours were only altered in the 5mg group, suggesting perhaps that a minimum level of hormonal priming is needed for this treatment to have an effect on sexual desire (Snoeren et al., 2011). Interestingly, the effects of this same combination in humans only had a positive effect in women with HSDD and showed no change in women who were of normal sexual functioning (van der Made et al., 2009).

As mentioned previously, DA may affect female sexual behaviour by acting as an intermediary for T. It is therefore possible that the initial administration of T that is effective in combination with PDE-5 inhibitors may result in an increase in DA release in the mPOA prior to the genital response effects seen due to prevention of cGMP breakdown. This combined effect may be important in setting up the timing mechanism that is so imperative for the display of female sexual behaviour in the rodent, so that first precopulatory behaviours may be increased followed by a switch in the mechanism due to DAR activity causing an inhibition of precopulatory behaviours and a display of lordosis, as seen in the model in Chapter 5. This particular treatment may only be effective in helping women with low excitation as a cause of HSDD, indicating that its effectiveness in female rats primed with EB-alone may be due to a lower than normal amount of DA transmission stimulated in the mPOA. In healthy women, or rats primed with EB+P, perhaps the baseline level of DA is high, such that the initial T administration increases levels too much, resulting in a spillover to DA D2R activation, and thus disrupting the timing mechanism and preventing an increase in sexual desire.

Dopamine Agonists

Finally, it should be noted that DA agonists such as apomorphine and the DA/NE reuptake inhibitor bupropion have also been tried as a treatment for increasing FSIAD. For example, bupropion administration has been found to increase sexual desire and potentially the frequency of sexual activity (Modell, May, & Katholi, 2000; Segraves, Croft, Kavoussi, Ascher, Batey, Foster, Bolden-Watson, & Metz, 2001; Segraves, Clayton, Croft, Wolf, & Warnock, 2004; Derzko, Elliott, & Lam, 2007). Apomorphine, meanwhile, has been shown to increase sexual desire, arousal, orgasm, and enjoyment, through both "as-needed" dosing and daily intake (Caruso et al., 2004). Interestingly, apomorphine has also shown to have an effect with women suffering from female orgasmic disorder, with administration increasing subjective arousal and lubrication, though not orgasm (Bechara, Bertolino, Casabe, & Fredotovich, 2004). Although

further research is required, it is plausible that the effects of these DA agonists are similar to the behavioural effects seen previously following DA agonists in female rats (Graham & Pfaus, 2010).

Conclusion

This thesis has examined whether behavioural differences previously observed that are dependent upon differences in DAR in the mPOA are caused by physiological changes to these receptors under different hormonal conditions. It was established that different hormonal administrations to the female rat causes changes to DAR in terms of indirect number located on neurons, protein quantities, and functional expression. In support of our previous behavioural findings (Graham & Pfaus 2010; Graham & Pfaus, 2012), administration of EB-alone results in a DA D2R-mediated system, as these females seem to have more DA D2R present and functional and activation of these receptors promote the onset of precopulatory behaviours. Administering P in addition to EB results in a switch to a DA D1R-mediated system that has more DA D1R available for binding, and activation of these receptors facilitates precopulatory behaviours. Additionally, it was found that these same hormonal manipulations also alter projections from the mPOA to areas important for the display of sexual behaviour, and some, but not all, of these projections have DAR available on them. These projections are expected to communicate information pertinent to the display of sexual behaviour, and are quite likely to be responsible for the coordination of different aspects responsible for the full display of female sexual behaviour.

Overall the physiological changes observed in this thesis have broad implications for the understanding of female sexual behaviour in the rat, and more generally in understanding the central role played by DA in the mPOA in female (and perhaps male) sexual desire. A potential mechanism has been established that may explain how separate systems controlling precopulatory behaviour and lordosis, as originally proposed by Ward and colleagues (1975), may be linked so that these behaviours can both be displayed throughout a copulatory bout. Previously proposed as a central processor for female sexual behaviour (e.g., Pfaus et al., 2015), the findings of the current thesis provide evidence of an exact mechanism within this area that integrates sensory and autonomic systems responsible for the full and integrated display of appetitive and consummatory sexual behaviours. This suggests that the mPOA, and DAR within it, may be the answer to a question originally proposed by Sherrington in 1906, and further

elaborated upon by Pfaff and colleagues (2006), as to what executes behavioural response transitions throughout the exhibition of female sexual behaviour.

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