The effects of endogenous and exogenous gonadal hormones on spatial navigation in women and in female rats.

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Abstract

The effects of endogenous and exogenous gonadal hormones on spatial navigation in women and in female rats.

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Gonadal hormones, both endogenous and exogenous, are implicated in cognition. Yet, the role of gonadal hormones in spatial navigation remains relatively unexplored. While navigating a maze either place memory or response memory can be used. The gonadal hormone 17β-estradiol affects which memory system female rats use during navigation, thus producing a *memory bias*. Across the menstrual cycle, women's memory bias is also altered. This thesis examined the role of endogenous and exogenous hormones in spatial navigation in both female rats and in women. It was shown that the endogenous hormone, progesterone promoted the use of response memory in females. It was also shown that the exogenous hormones used in hormonal contraceptives impact memory bias in females. However, the impact of these hormones on spatial navigation was different in rats than what was observed in humans. It was also shown that both endogenous and exogenous hormones have different effects depending on whether they are administered alone or in combination. Both endogenous and exogenous gonadal hormones are involved in memory bias during spatial navigation in females.

Contributions of Authors

Chapter 1. Progesterone rapidly alters the use of place and response memory during spatial navigation in female rats.

Jesse M. Lacasse. Designed and conceived experiment, conducted behavioural study, ran ELISAs, collected data and ran all statistical analyses, wrote and edited manuscript.

Smita Patel. Helped conduct behavioural study, helped with ELISAs.

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Wayne G. Brake. Helped to design and conceive experiment. Helped to write and edit manuscript.

Chapter 2. Modeling hormonal contraception in female rats: A framework for studies in behavioral neurobiology.

Jesse M. Lacasse. Conceived animal model, conducted literature review, ran ELISAs, created figures and tables, wrote and edited manuscript.

Eamonn Gomez-Perales. Collected microscope images of vaginal cytology.

Wayne G. Brake. Helped conceive animal model. Helped write and edit manuscript.

Chapter 3. Combined effects of the contraceptive hormones, ethinyl estradiol and levonorgestrel, on place and response memory in gonadally-intact female rats.

Jesse M. Lacasse. Designed and conceived experiment, conducted behavioural study, ran ELISAs, collected data and ran all statistical analyses, wrote and edited manuscript.

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Conall E. Cionnaith. Helped conduct hormone injections, helped with ELISAs.

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Wayne G. Brake. Helped to design and conceive experiment. Helped to write and edit manuscript.

Chapter 4. Biological sex, gonadal hormones, and oral contraceptives impact spatial navigation in a 3-dimensional virtual Hex maze task.

Jesse M. Lacasse. Designed and conceived experiment, ran study participants, collected data and ran all statistical analyses, wrote and edited manuscript.

Sean Devine. Helped run study participants. Helped set-up the computer-based task.

Thomas D. Ferguson. Supplied and helped set-up the computer-based task.

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Benjamin Eppinger. Helped to design and conceive experiment. Provided lab-space for study to take place.

Wayne G. Brake. Helped to design and conceive experiment. Helped to write and edit manuscript.

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List of Abbreviations

17β-HSD: 17β-hydroxysteroid dehydrogenase
AD: Alzheimer's disease
ALLO: Allopregnanolone
AR: Androgen receptors
CBG: Corticosteroid-binding globulin
CMA: Chlormadinone acetate
COC: Combined oral contraceptives
CPA: Cyproterone acetate
CYP: Cytochromes P450
DHEA: Dehydroepiandrosterone
DHT: Dihydrotestosterone
DNG: Dienogest
DRSP: Drospirenone
DSG: Desogestrel
DS: Dorsal striatum
E: Estrogens
E1: Estrone
E2: 17β-estradiol
EB: Estradiol benzoate
EE: Ethinyl estradiol
ELISA: Enzyme linked immunosorbent assay
ENG: Ethonogestrel
EPM: Elevated plus-maze
ER: Estrogen receptors
ERα: Estrogen receptor α
ERβ: Estrogen receptor β
EV: Estradiol valerate
fMRI: Functional magnetic resonance imaging
FSH: Follicle stimulating hormone
GABA: γ-aminobutyric acid
GDX: Gonadectomized
GnRH: Gonadotropin-releasing hormone
GTD: Gestodene
HC: Hormonal contraceptives
HPC: Hippocampus
HPG: Hypothalamic-pituitary-gonadal
HRT: Hormone replacement therapy
IUD: Intrauterine device
LC/MS: Liquid chromatography/mass spectrometry
LH: Luteinizing hormone
LNG: Levonorgestrel
MC: Menstrual cycle
MPA: Medroxyprogesterone acetate
mPFC: Medial prefrontal cortex
mPR: Membrane progesterone receptor
MWM: Morris water maze
NC: Naturally cycling
NET: Norethisterone
NETA: Norethisterone acetate
NG: Norgestrel
NOMAc: Nomegestrol acetate
NOR: Norgestimate
NST: Nestorone
OC: Oral contraceptive
OLMT: Object-location memory task
OVX: Ovariectomized
P: Progesterone
PET: Positron Emission Topography
POP: Progestin-only pill
PR: Progesterone receptors
RAM: Radial arm maze
RBA: Relative binding affinity
ROA: Route of administration

GPER: G protein-coupled estrogen receptor 1

T: Testosterone

vMWM: Virtual Morris water maze

vRAM: Virtual radial arm maze

S.c.: Subcutaneous

SHBG: Sex hormone binding globulin

General Introduction

Frame of reference

In 1946, Tolman and his colleagues established that rats could use more than one memory system while navigating a maze (Tolman et al., 1946). Tolman et al. (1946) differentiated between at least two such memory systems: place memory and response memory. Place memory entails using a 'cognitive map' in order to navigate. Response memory entails using well-practiced motor patterns in order to navigate (Goodman, 2021). Human analogues to place and response memory are coined allocentric and egocentric strategies, respectively (Chersi and Burgess, 2015; Ekstrom and Isham, 2017). In both rodents and humans, males and females use place/response memory or allocentric/egocentric strategies to varying degrees during navigation. For example, after initial exposure to a maze environment male rats use place memory, but with practice, shift to using response memory only (Chang and Gold, 2003). However, in female rats the same pattern of results is not so persistent. In studies of human navigation, men predominantly use an allocentric strategy, while women tend to use an egocentric strategy (Andersen et al., 2012; Sandstrom et al., 1998; Spriggs et al., 2018). A possible explanation for these differences between males and females is their different exposures to gonadal hormones, endogenous or exogenous.

Korol et al. (2004) demonstrated the influence of gonadal hormones on place and response memory. They showed that female rats used different memory systems depending on their estrous cycle phase. Several studies that followed demonstrated that when levels of circulating 17β-estradiol were low, female rats biased towards using response memory. In contrast, when 17β-estradiol levels were high, they biased towards using place memory (Almey et al., 2014; Hussain et al., 2016a; Korol and Kolo, 2002; Quinlan et al., 2008, 2013). These studies clearly demonstrated a role for gonadal hormones such as 17β-estradiol in biasing female rats towards using either place or response memory. The concept that there is a bias to

predominantly engage one memory system over another depending on circulating hormone levels in females has since been coined *memory bias* (Hussain et al., 2014; Quinlan et al., 2013).

The findings from rodent studies prompted similar research in humans which uncovered the influence menstrual cycle phase on memory bias in women but emphasized the importance of considering progesterone (Hussain et al., 2016b). Progesterone had only been examined in the context of memory bias in a single experiment in rats (Korol and Pisani, 2015) and therefore, its role required further attention. In addition, although the menstrual cycle was shown to play an important role in memory bias in naturally-cycling women. However this excluded women taking oral contraceptives who have a different hormonal milieu (Hampson, 2023).

The aim of this thesis was to further examine the role of endogenous and exogenous hormones in spatial navigation in both female rats and in humans. This thesis examined 1) whether progesterone influences memory bias; 2) whether exogenous hormones such as those used in oral contraceptives affect memory bias; 3) how biological sex, gonadal hormones, and oral contraceptives can impact spatial navigation in humans.

The general introduction of this thesis is largely comprised of a previously published review manuscript.

Sex differences in spatial navigation: ***The role of gonadal hormones.***

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Abstract

In the past decade studies have illuminated a more nuanced pattern in sex differences in navigating an environment and the bias to use one or another memory system to solve a navigational task. This review focuses on two types of memory in rodents; place/spatial memory and response/habitual memory. These two types of memory are affected by levels of gonadal hormones such as testosterone, estrogens, and progesterone. Studies on similar types of memory in humans also show sex differences, albeit the influence of hormones in women do not match the rodent research. Hormone levels are rarely measured when testing sex differences in humans. Thus, we need more research that measures hormones while also measuring sex differences in these memory systems important for navigation.

Key Words: estrogens; progesterone; spatial memory; response memory; hippocampus; prefrontal cortex; dorsal striatum; testosterone; allocentric; egocentric

Introduction

Levels of gonadal hormones (e.g. androgens, estrogens, and progesterone) fluctuate in both sexes (Chong et al., 2015; Roney and Simmons, 2017). Estrogens and progesterone fluctuate monthly in women (Catenaccio et al., 2016) and testosterone fluctuates seasonally in men (Demir et al., 2016). Hormone levels also change across the lifespan (e.g. puberty, pregnancy, and menopause; Catenaccio et al., 2016). Accumulating rodent research suggests that activational effects of gonadal hormone levels can account for dramatic differences in navigation strategy and overall performance on certain tasks of spatial navigation (Almey et al., 2015; Frick et al., 2015; Wagner et al., 2018). Relating to early work by Tolman, Ritchie, and Kalish (1946), the term *strategy preference* refers to differences in the navigational approaches used by rodents while navigating a maze environment. Measures of performance generally vary from task to task. In maze navigation tasks *performance* is usually measured by the amount of time taken to learn and/or properly navigate a spatial environment. The purpose of this review is to examine sex differences in the recent rodent and human literature highlighting the nuanced ways in which hormonal fluctuations affect strategy preference and performance in spatial navigation.

Spatial/place vs. response/habitual memory in rodents.

Spatial memory, otherwise known as place memory, refers to making associations with landmarks in the spatial landscape to form a cognitive map. Spatial memory is used both when navigating an environment and when solving object spatial recognition tasks (Tolman et al., 1946). Often in these tasks, rodents are trained to navigate a maze toward a specific goal or reward. Using spatial memory is thought to be more cognitively demanding than is response memory, but it is useful in a novel environment where the general landscape can be used to find the target destination.

In addition to spatial memory, rodents may also use response or habitual memory when navigating a spatial environment. Response memory is an approach to solving a spatial

navigation task that relies predominantly on internal cues such as habitual body turns (Tolman et al., 1946). A response strategy is thought to offer an efficient way to navigate and may be advantageous once the rodent has become sufficiently accustomed to an environment. To dissociate between spatial and response memory use, one method is to train rats to repeatedly enter one arm in a T-maze in order to retrieve a reward. Once a rat is trained to consistently enter that arm, the maze is rotated 180 degrees during a probe test. If a rat turns in the direction it was always trained to during the probe, it is scored as using response memory. Contrarily, if it turns towards the same spatial location in the room where it was rewarded previously, it is scored as using place memory (Fig. 1).

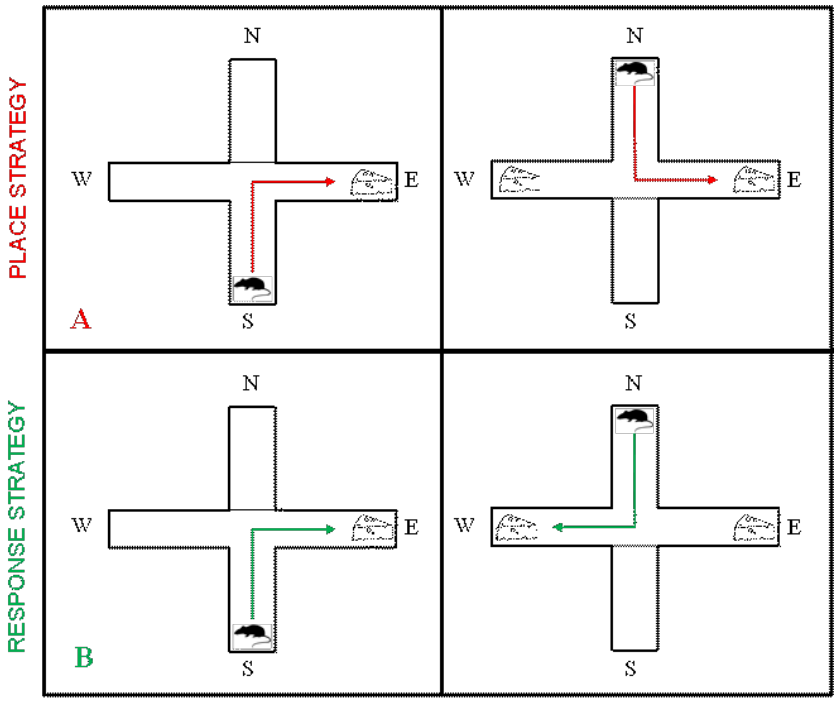

Figure 1. Illustration of how place and response navigation are dissociated in an ambiguous T-maze. In panel A, the rat is consistently trained to turn right, and on the probe trial turns left towards the same spatial location - indicating it used place memory. Panel A illustrates spatial or place memory being used. In panel B, the rat is trained to consistently turn right, and on the probe trial turns right, the same direction it had habitually been trained to turn indicating it used response memory.

Spatial and response memory have been linked to two primary brain regions in rodents: the hippocampus (HPC), and the dorsal striatum (DS), respectively. McDonald and White (1993) identified a link between the HPC and place memory noting that damage to the HPC led

to deficits in spatial learning and memory. Likewise, damage to the DS impaired performance on a response memory task without any motor, sensory or motivational impairments. Additionally, while hippocampal damage was detrimental to performance on a place memory task, it increased the speed at which response-based tasks were learned, vice versa (White and McDonald, 2002). Taken together, these findings demonstrate that hippocampal-based spatial/place memory and striatum-based response/habitual memory act competitively while navigating an environment.

The role of estrogens and progesterone in spatial and response memory in female rodents.

Korol et al. (2004) first showed that female rats navigating a T-maze are biased either toward a spatial or a response memory strategy across the estrous cycle; the rodent equivalent of the human menstrual cycle. While in the pro-estrus phase, when estrogens are high, female rats tend to bias towards using spatial memory. On the other hand, during the estrus phase, when estrogens are lower, female rats are more biased towards using response memory (Korol et al., 2004). Hippocampal volume has been shown to vary across the estrous cycle of mice such that it is greatest during pro-estrus and lesser during estrus. These shifts in hippocampal volume are correlated with a preferential use of spatial memory when hippocampal volume is increased and response memory when it is diminished (Qiu et al., 2013). In fact, direct infusions of one of the most abundant estrogens, 17β-estradiol (E2) into the HPC of female rats enhanced place learning strategies and had no effect on response learning (Zurkovsky et al., 2007, 2006). Contrarily, infusions of E2 into the DS impaired performance when the task required response memory, but had no apparent effects when the task demanded use of spatial memory (Zurkovsky et al., 2011, 2007). Recent findings have shown that the medial prefrontal cortex (mPFC) regulates which of these two memory types will be used in female rats. Almey et al. (2014) showed that micro-infusions of E2 directly into the mPFC promotes a shift from the use of striatal-based response memory to a hippocampal-based spatial memory. Indeed mPFC

neurons are activated in response to a switch from a place to a response strategy but not in response to behavioural or task contingencies (Rich and Shapiro, 2009). These data indicate that the mPFC is also important for memory bias and is sensitive to E2.

In order to remove cyclic variability and to dissociate the effects of individual hormones on strategy preference, E2 and P levels have been manipulated in ovariectomized rats. Rats that were ovariectomized and administered a high dose of E2 showed a bias towards spatial memory, whereas those replaced with low E2 were biased toward using response memory (Quinlan et al., 2013). In a recent study, Lacasse et al. (2018) administered either low E2, high E2, or high E2+P to ovariectomized female rats. As shown previously, rats given low E2 were biased towards using response memory and those given high E2 were biased towards spatial memory. However, rats injected with high E2+P showed a bias towards response memory. Thus, it would appear that P might be reversing the shift in bias which occurs between low- and high-levels of E2. With regard to the role of P in spatial navigation performance, the literature is unclear.

Some studies have outlined the positive effects of P on spatial performance, while others have shown disruptive effects of P (for review see Barros et al., 2015). Additional difficultly in interpreting the results of these studies arises from not being able to dissociate the effects of P from the effects of its metabolites (Barros et al., 2015; Schumacher et al., 2014). For example, allopregnanolone (a P metabolite) has been shown to bind to $GABA_A$ receptors, subsequently affecting GABAergic transmission (Schumacher et al., 2014). The Morris water maze (MWM) is an animal task often used for examining spatial navigation which typically requires a rodent to swim in a pool with opaque water in order to learn the location of a hidden platform. Allopregnanolone has been shown to impair performance when administered to rodents shortly before being tested in a MWM (Johansson et al., 2002). Still, further research is needed to understand the effects of P on spatial navigation and disassociate P from the effects of its metabolites.

The role of testosterone in spatial and response memory in male rodents.

Chang and Gold (2003) found that when male rats were tested in a T-maze and tasked to find a reward, they began by using spatial memory. However, after repeated trials when they had become familiar with the environment, they eventually shifted to using response memory (Chang and Gold, 2003). Yet, more recent evidence proposes that a bias towards using spatial or response memory in male rats may be modulated by circulating androgen levels (Jacome et al., 2016; McConnell et al., 2012; Spritzer et al., 2013; Wagner et al., 2018).

Recent experiments have illustrated that testosterone may regulate the bias between spatial and response memory in a dose-dependent manner (Spritzer et al., 2013; Wagner et al., 2018). Low doses of T biased rats towards using response memory, whereas, higher doses promoted a bias towards using spatial memory (Spritzer et al., 2013). Regarding navigational performance, administration of T to gonadectomised male rats reversed an impairment in spatial memory caused by castration in an object-location memory task (OLMT; McConnell et al., 2012; Moghadami et al., 2016). The OLMT requires a rodent to learn and subsequently recall the spatial location of an object after an initial training trial. Doses of 0.125mg and 0.500mg (but not 0.200mg or 1.00mg) of T improved spatial memory performance in a radial arm maze (RAM) and in an OLMT compared to castrated controls (Wagner et al., 2018). Similarly, Jacome et al. (2016) showed that an acute dose of T rapidly improved performance of castrated male rats in an OLMT. However, it remains unclear whether the findings above are exerted via Ts action, or whether it is through T being aromatized into E2. McConnell et al. (2012) showed that administering T or E2 both improved performance on an OLMT in male castrated rats. Findings such as these illustrate the need for further research into the underlying mechanisms regarding spatial memory in rats.

Allocentric and egocentric navigation in humans

The literature on human spatial navigation uses somewhat different terminology than the animal literature when referring to how humans navigate a spatial environment. The terms

“egocentric” and “allocentric” navigation are used to describe the perspective (e.g., first person or third person) taken by the navigator (Hartley et al., 2003; van Gerven et al., 2012). During egocentric navigation, the first person perspective is taken and the navigator is guided by internally based cues (Hartley et al., 2003; Hussain et al., 2014; Fig. 2a). Allocentric navigation is a cognitively demanding, yet flexible, third person way of navigating by using the spatial relationship between landmarks to orient oneself. In allocentric navigation, a cognitive-map of the environment is created by forming spatial relationships between objects and landmarks (O’Keefe and Nadel, 1978; Fig. 2b).

One cannot directly compare between rodents and humans - as cognition and the neural circuitry involved are more complex in humans than rodents. Nonetheless, functional magnetic resonance imaging (fMRI) has since confirmed the involvement of the same two brain regions described in the rodent literature (viz. the HPC and the DS) are also involved in allocentric and egocentric navigation in humans. When a virtual environment was designed to promote use of an allocentric perspective during navigation, the HPC was shown to have increased activity (Maguire et al., 1998). On the contrary, if the virtual environment promoted use of egocentric navigation (e.g. no spatial landmarks), it was the DS (a.k.a. caudate nucleus) that showed increased activation (Hartley et al., 2003). These findings have been replicated using fMRI on humans while navigating a virtual eight-arm radial arm maze task (Bohbot et al., 2004; Iaria et al., 2003). For detailed review of hippocampal and striatal contributions to human spatial navigation see Chersi and Burgess (2015).

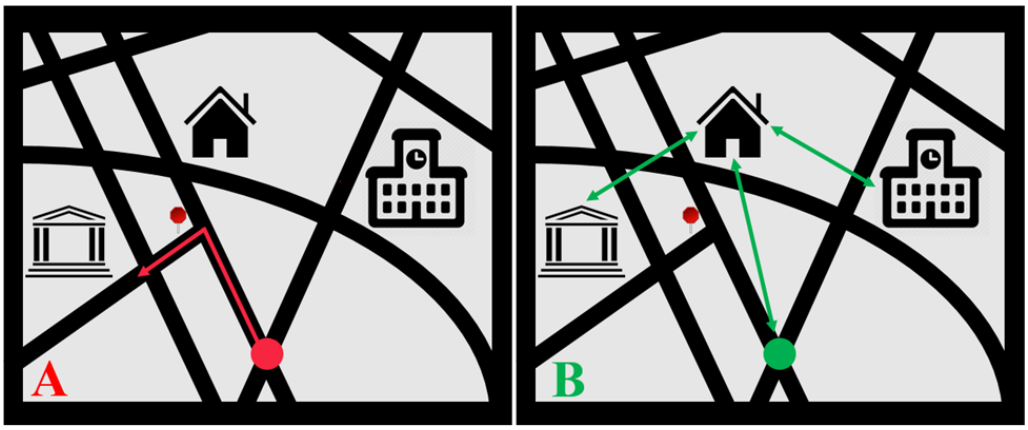

Figure 2. Illustration of the difference between egocentric and allocentric navigation. Panel A illustrates egocentric navigation, showing the individual navigating based on internal and landmark-based cues e.g. head straight and turn left at the stop sign to reach the bank. Panel B illustrates allocentric navigation where the individual refers to spatial relationships between landmarks, and where the individual is relative to those landmarks.

Sex differences in human spatial navigation

Researchers have been interested in sex differences in spatial abilities for decades (Linn and Petersen, 1985). More recently spatial navigation tasks from the animal literature have been adapted to a virtual setting to be used in human research. Virtual editions of the MWM (vMWM) have been designed for human testing; asking participants to navigate a virtual pool to find a hidden platform using a joystick. The use of landmarks and cues can also be manipulated by adding or removing them to help differentiate between allocentric and egocentric navigation. The most consistent finding within the vMWM is that men tend to locate the hidden platform more quickly than do women. Men also tend to travel less total distance before locating the hidden platform (Daugherty et al., 2015; Gazova et al., 2013; Korthauer et al., 2017; Mueller et al., 2016, 2008; Nowak and Moffat, 2011; Piber et al., 2018; van Gerven et al., 2012; Woolley et al., 2010). During the probe trial, men tend to spend more time in the goal quadrant than do women (Daugherty et al., 2015; Korthauer et al., 2017; Mueller et al., 2008; Nowak et al., 2014; Nowak and Moffat, 2011; van Gerven et al., 2012). Yet, other studies have not found sex differences in performing this task (Mueller et al., 2016; Piber et al., 2018; Rodgers et al., 2012; Sneider et al., 2015). It should be noted that in some of these experiments, men outperformed

women only in the initial trials. However, over time and as the task was learned, both sexes performed equally well. Interestingly, such disappearing sex differences after acquiring the MWM are parallel to what has been observed in rodents (for review see Simpson and Kelly (2012).

The radial arm maze (RAM) typically has eight arms extending from a central node. Although it can be used in many ways, one may be that certain arms are baited with a reward and others not. Performance can be measured as time taken to retrieve the rewards and/or wrong arms entered. In rodent models, males show overall better performance than do females on the RAM (for review see Simpson and Kelly, 2012). The RAM has also been adapted into a virtual edition (vRAM) for humans. In the vRAM, the participant sees the center of an arena with usually eight arms that they can virtually enter. While learning the environment, the participant navigates the vRAM to discover which arms have rewards in them. As in the MWM, landmarks and spatial cues can be manipulated in the vRAM in order to differentiate between allocentric and egocentric navigation.

Early studies in humans reported no sex differences in performance in the vRAM (Astur et al., 2004; Levy et al., 2005), however, more recent studies have. Andersen, Dahmani, Konishi, and Bohbot (2012) showed that women took longer than men to complete the vRAM task (viz. locating all correct goal arms). Additionally, women tended make more errors than men. They entered the same arms more than once, and entered incorrect arms more often. If there were landmarks present during the acquisition of the task, women's, but not men's, performance subsequently worsened if those landmarks were removed (Andersen et al., 2012). The preferential use of landmarks by women was also observed by Piber et al. (2018) using a vMWM. Yet, the study by Andersen et al. (2012) used only seven participants which limits our ability to draw any firm conclusions. It should also be noted that these sex differences appear to persist after controlling for prior video game and joystick experience (Daugherty et al., 2015;

Korthauer et al., 2017; Mueller et al., 2016, 2008; Nowak and Moffat, 2011; Piber et al., 2018; van Gerven et al., 2012).

Whether there is a sex difference in the preferential use of allocentric and egocentric navigation is still equivocal. In 2004, a review of the past two decades of research on sex differences in spatial orientation concluded that males predominantly used allocentric, while women used egocentric, navigation styles (see Coluccia and Louse, 2004). Across all types of navigation tasks, recent studies have concluded that males prefer an allocentric navigational approach (Astur et al., 2016; Spriggs et al., 2018). Additionally, these studies report that women show a slight preference for egocentric navigation (Spriggs et al., 2018) or no preference (Astur et al., 2016). Others have shown that men and women may both show an equal preference for using allocentric navigation (van Gerven et al., 2012). Yet, multiple studies have concluded that there were no statistically significant relationships between sex and strategy preference (Andersen et al., 2012; Bohbot et al., 2012; Gazova et al., 2013; Rodgers et al., 2012). As we have seen in the preceding studies, we are limited in our abilities to draw conclusions about sex differences as they relate to strategy preference in humans. In some cases, males prefer allocentric versus egocentric navigation, while in other cases, there appears to be no clear divergence in navigation preference based on sex.

As established, how a rat performs within a spatial navigation task can be sensitive to fluctuations in gonadal hormones. Taken together, human studies may be underrepresenting true differences in how men and women navigate a spatial environment by not accounting for variations in hormone levels. While acknowledging that rodent models do not necessarily reflect the complexity of human cognition, the animal literature demonstrates the importance of considering fluctuations in hormone levels.

More recently, a few studies have measured hormone levels when testing spatial navigation in humans. Hussain et al. (2016b) divided women into three conditions based on their menstrual cycle phase: follicular (low E2 / low P), ovulatory (high E2/ low P), or luteal

(moderate E2/high P). Women in both the follicular and ovulatory phase predominantly used an egocentric navigational approach. However, in the luteal phase when levels of P peaked, women tended to use an allocentric approach in the vRAM. These results are different from what we found in a rodent study (Lacasse et al., 2018). However, the four-day estrous cycle of the rat differs from the human menstrual cycle, which may account for different responses to circulating gonadal hormones. Overall, Hussain et al.'s (2016) findings suggest that women may alternate between allocentric and egocentric navigation across different phases in the menstrual cycle. Conversely, others found no effect of the menstrual cycle on navigation strategy preference (Scheuringer and Pletzer, 2017). However, this study used a paper-pen style 2D spatial navigation task, thus limiting the ability to generalize to navigation of a 3D environment.

Circulating T levels may also be predictive of performance for both men and women in a spatial navigation task (Burkitt et al., 2007; Mueller et al., 2016). However, no clear relationship between T and spatial performance has been established. Some have shown T improves performance in spatial navigation tasks (Burkitt et al., 2007; Mueller et al., 2016). Others have demonstrated that it was low levels of T in particular which promote better navigational performance in a vMWM (Nowak et al., 2014). At least one study found no relationship between T levels and spatial navigation performance (Pintzka et al., 2016). The evasiveness of a clear pattern of results illustrates the complexity of Ts effects on spatial navigation in humans.

Conclusions and future studies.

The research above has outlined both in rodents as well as in humans, that levels of gonadal hormones should be taken into account as an essential variable when looking at strategy preference and performance in spatial navigation tasks. Future studies could clarify previous work on spatial navigation by considering hormonal fluctuations; for example: by avoiding clustering results from all women into a single group. Instead, strategy preference or navigation performance should be understood according to the participants cycle phase (e.g.

follicular, ovulatory, luteal; see Becker et al., 2005). Likewise, it is important to control for whether or not female participants are using hormonal contraceptives; particularly which type and dose, as their hormonal profiles will differ significantly user-to-user and to those of naturally cycling women (Pletzer and Kerschbaum, 2014).

Studies of aging populations should also take into consideration whether or not the individual (of either sex) is using hormonal replacement therapy (HRT). Older individuals using HRT may show a different pattern of results than those without any hormone replacement (see Galea et al., 2017 for review). As such, studies investigating the effects of aging on spatial navigation should consider the hormonal status of the participant. Moving forward, future studies may also consider experimentally manipulating hormone levels by administering transdermal patches or gels as seen in (Carré et al., 2017). This would allow for careful dissociation of the effects of individual hormones at varying doses on strategy preference and navigational performance.

Thesis aims

The overarching aim of this thesis is to further elucidate the role of endogenous and exogenous steroid hormones in memory bias, both in female rats and in humans. As previously described, gonadal hormones such as E2 clearly influence whether a female rat is biased towards using place or response memory. In humans, however, the role of gonadal hormones is less straightforward. Hussain et al., (2016b) showed that in the early follicular phase when E2 were low and during the ovulatory phase when E2 levels were high, there was a bias to use an egocentric strategy. This contrasts what is observed in female rats. Only when progesterone levels rise during the mid-luteal was there a shift towards the use of an allocentric strategy in women. Thus, the role of P in regulating memory bias required further investigation.

The first aim of this thesis was to expand our understanding of the role of P in memory bias in female rats. Thus, in Chapter 1, we demonstrate that P does impact memory bias, and that the timing of its administration is an important consideration.

The next aim of this thesis was to investigate how exogenous hormones, such as those used in hormonal contraceptives might impact memory bias in female rats. However, first an animal model of human hormonal contraception with appropriate ecological validity had to be developed. Therefore, in Chapter 2 previous studies on modelling hormonal contraceptives in female rats were thoroughly reviewed. In addition, the development of our own model of hormonal contraception in female rats is outlined.

The aim of Chapter 3 was to use our model of hormonal contraceptives in female rats to investigate memory bias. By so doing, it was demonstrated that the exogenous hormones contained in hormonal contraceptives also influence memory bias in female rats.

The final aim of this thesis was therefore to examine the effects of both endogenous and exogenous gonadal hormones on memory bias in humans. Thus, in Chapter 4 we demonstrate that both endogenous and exogenous gonadal hormones can impact memory bias and navigation performance in humans. These findings highlight the importance of considering endogenous and exogenous hormones when studying spatial navigation.

Chapter 1: Progesterone rapidly alters the use of place and response memory during spatial navigation in female rats.

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Abstract

17β-estradiol (E2) and progesterone (P) influence place and response memory in female rats in spatial navigation tasks. Use of these memory systems are associated with the hippocampus and the dorsal striatum, respectively. Injections of E2 result in a well-established bias to use place memory, while much less is understood about the role of P. A total of 120 ovariectomized female rats were tested within a dual-solution T-maze task and treated with either low E2 (*n*=24), high E2 (10μg/kg; *n*=24), or high E2 in combination with P (500μg/kg) at three time points before testing: 15 minutes (*n*=24), 1 hour (*n*=24), and 4 hours (*n*=24). Given alone, high E2 biases rats to the use of place memory, but this effect is reversed when P is given 1 hour or 4 hours before testing. This indicates that P may be playing an inhibitory role in the hippocampus during spatial tasks, which is consistent with past findings. Our findings show that P acts rapidly (within an hour) to affect performance during spatial tasks.

Keywords: Progesterone, 17β-estradiol, hippocampus, dorsal striatum, T-maze, spatial navigation, multiple memory systems.

Introduction

While navigating a maze, rats can use either place or response memory (Tolman et al., 1946). The dual-solution task is a variation on the plus-maze designed to test these two distinct memory systems (Blodgett and McCutchan, 1948; Restle, 1957). The use of *place* memory is associated with the hippocampus (HPC) while the use of *response* memory is linked to the dorsal striatum (DS; see Goodman, 2021 for review). Moreover, lesions of the HPC results in the predominant use of the DS, while inactivation of the DS results in the predominant use of HPC (Packard et al., 1989; Packard and McGaugh, 1996).

Place and response memory interact competitively. This is demonstrated by studies showing that inactivation of the HPC actually enhances acquisition of striatal-based navigation tasks (McDonald and White, 1994). The opposite is true for inactivation of the DS: it enhances performance on navigation tasks engaging the HPC (Asem and Holland, 2015; Chang and Gold, 2004; Gornicka-Pawlak et al., 2015). Administering rats intra-ventricular infusions of beta-amyloid protein, a protein associated with Alzheimer's disease (AD), leads to the greater use of the striatal-based response memory in a dual-solution task (Ammassari-Teule et al., 2002). Similar findings have been reported from studies using a transgenic mouse model of AD (Middei et al., 2006, 2004). Thus, in situations where the HPC is structurally or functionally impaired, the memory systems subserved by the DS are more readily engaged.

In female rats, a *memory system bias* between place or response memory depends on the circulating levels of ovarian hormones such as 17β-estradiol (E2). For example, when circulating E2 levels are low, rats show a response memory bias. Yet, when E2 levels are high, rats show a place memory bias (Quinlan et al. 2013; Hussain et al. 2016; Korol and Pisani, 2015; Quinlan et al., 2008; Korol et al., 2004; Korol & Kolo, 2002). Moreover, systemic and local infusions of E2 enhance acquisition on place memory tasks and impair it on response memory

tasks (see Korol and Wang, 2018 for review). Progesterone (P) has not been as extensively studied within the dual-solution task but appears to impair performance on other spatial tasks such as the Morris Water Maze (MWM) or the Radial Arm Maze (Barros et al., 2015; Bimonte-Nelson, 2004; Bimonte-Nelson 2003; Chesler and Juraska, 2000; Warren and Juraska, 1997). Using a dual-solution T-maze task, Korol and Pisani (2015) observed that ovariectomized rats show a response memory bias, while those that were ovariectomized and given estradiol benzoate (EB) show a place memory bias. Yet, rats that received a combination of both EB and P are biased to using response memory, the same as was seen in the OVX only condition. However, it is unknown how quickly P exerts this effect.

Here, we expanded on past work by examining whether P impacted memory bias rapidly or over the course of hours. Female rats were ovariectomized and administered either low E2, high E2, or high E2 with P at three time points: 15 min, 1h, or 4h before testing. We hypothesized that rats given low E2 would show a response memory bias, while those given high E2 would show a place memory bias, as shown in previous work (Quinlan et al. 2013; Hussain et al. 2016; Korol and Pisani, 2015; Quinlan, 2008; Korol et al., 2004; Korol & Kolo, 2002). We also hypothesized that P would alter memory system bias such that rats given P would use response memory as shown by Korol and Pisani (2015).

Methods

Test Subjects. A total of 120, two-to three-month-old female Long Evans rats weighing ~220-240g on arrival were used. 89 of these rats arrived from Charles River, Canada and 31 were bred internally from the Animal Care Facility at Concordia University. Rats were housed in pairs until they were ovariectomized and food restricted and then they were housed individually in a single shoe-box plastic cage (dimensions: 25.5 cm wide x 46.6 cm long x 21.6 cm high) under a 12h reverse light-dark cycle (2000 to 0800) and drank water from a plastic receptacle.

They had *ad libitum* access to chow (Teklad Global Rodent Diet ®). This chow has been formulated to exclude items which may contain phytoestrogens such as soybean meal. However, it does include soy oil which may or may not include phytoestrogens. Food restriction began one week after surgeries and entailed maintaining weights at 80% of free-feeding levels (~15-20 grams daily). These procedures were in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the Concordia Animal Research Ethics Committee.

Ovariectomy and Hormone Replacement. All rats remained gonadally intact during the handling and maze habituation phases. Rats were then ovariectomized one week before maze acquisition training began, which allowed 5-6 days of recovery post-surgery. Ovariectomy surgeries were carried out as described in previous work (Almey et al., 2013). During the OVX surgery, a capsule containing 5% E2 in cholesterol was implanted subcutaneously into each rat. E2 capsule preparation was as described in previous work (Almey et al., 2013). During the maze acquisition phase, no additional hormones were administered. One day before testing, each rat was randomly assigned to one of five hormone treatment conditions: (1) low E2 (*n* =24), (2) high E2 (*n* =24), or high E2 + P (*n* =24) with P being administered at either (3) 15min, (4) 1h, or (5) 4h before the probe trial.

Twenty-four hours before testing, rats receiving low E2 were given a vehicle injection (sesame oil, 0.1ml, s.c.) while those in all other conditions received a single injection of E2 (10μg/kg, s.c.; Sigma Chemical Co.). On test day, rats treated with low E2 and those treated with high E2 received a second vehicle injection 4h before testing to control for the injections given to those receiving P. Rats in the remaining E2 + P conditions received an injection of P (500μg/kg, s.c., Sigma Chemical Co.) either 15min, 1h, or 4h before testing. The E2 capsules have been shown to result in E2 levels that would be expected during the diestrus phase (Almey et al., 2013). The dosage for the high E2 injection was chosen as it has been shown to result in

levels of E2 observed in pro-estrus (Almey et al., 2013). The dosage for P was selected as it has been used previously in similar work (Korol and Pisani, 2015; Chesler and Juraska, 2000). It is also the same dose that is used for facilitating estrogen-induced estrus (Edwards, Whalen, and Nadler, 1968).

Behavioural Training/Testing.

Apparatus. Training and testing were carried out within a black Plexiglas T-maze which was placed on a table 1m above the floor. The T-maze was comprised of grey walls (28cm high), a stainless-steel grid floor, transparent Plexiglas ceiling panels, a start arm (75cm long), and two target arms (each 75cm long), which were each positioned at a 90° angle to the start arm. The start arm contained a sliding door which obstructed the first half of the arm, this created a start chamber. The target arm contained a small stainless-steel cup containing a Froot Loop (Kellogg's ™) at its end. Froot Loops were placed below the grid floor throughout the entire maze, in order to avoid the use of odor cues during acquisition and testing. Two additional sliding doors separated the target arms from the choice point of the start arm and could be closed to prevent the rats from leaving their chosen target arm once they were inside. An additional arm (identical in dimensions to the start arm) was added to the T-maze to form a plus-shape maze. Another sliding door closed off access to this fourth arm of the maze at all times, except during probe testing. The T-maze was situated in a room dimly lit with overhead red fluorescent lamps and a lamp facing the ceiling (40 W light bulb). Experimenters stood in a specified region located two feet from the plus-maze, serving as a spatial cue. Other spatial cues included cupboards and colorful posters on the walls.

Training. All rats were handled daily (for approximately 5 days) upon arrival from the colony to familiarize them with the people testing them (see Fig. 1a for training timeline). All rats were then subjected to 3 days of 10 min habituation sessions per day within the T-maze in order

for them to become familiar with the environment. This was followed by 5-7 days of 5 min maze acquisition sessions where the rats were required to seek out the Froot Loops (1/4 of a Froot Loop) baited in each of the goal arms. During these 5 min sessions, all sliding doors were opened. Following the habituation phase, the rats were ovariectomized before beginning 21 days of maze training. Five days after surgeries, training began. Rats were then randomly assigned an arm (right or left arm), and then trained to enter only that arm in order to receive a food reward. The reward arm was held constant in relation to the spatial cues in the room. Behavioural training sessions were carried out 7 days a week, started at approximately 1200-1300h, and lasted until 1500-1600h.

Every day for 21 days, each rat was given 10 choice trials where they were placed in the start arm and were allowed to choose either the right or left arm. Once the rat had chosen an arm and entered it, the guillotine door corresponding to that arm was closed behind it. Once the rat had eaten the food reward (or investigated the empty bowl in the case of choosing the incorrect arm) they were removed from the maze. If the rats chose the correct arm 8 out of 10 times (80%) for three consecutive days, they were considered to have met criteria for testing.

Testing. Test day began at 0800h when each rat was administered its respective hormone or vehicle, 15 min, 1h, or 4h later each rat was given ten choice trials to confirm criterion was still met, followed by a single probe trial. In the probe trial, the rat was placed in the opposing start arm 180˚ (opposite) to the original start arm. Access to the original start arm was fully closed off, and the rat was allowed access to the same two goal arms but approaching from the opposite side of the maze. The rat was considered to be using place memory if it entered the same arm where it received the reward during training. If instead the rat turned in the direction it was trained (e.g., the rat was trained to turn right, and when the maze was rotated, it still turned right) it was scored as using response memory (see Fig. 1b).

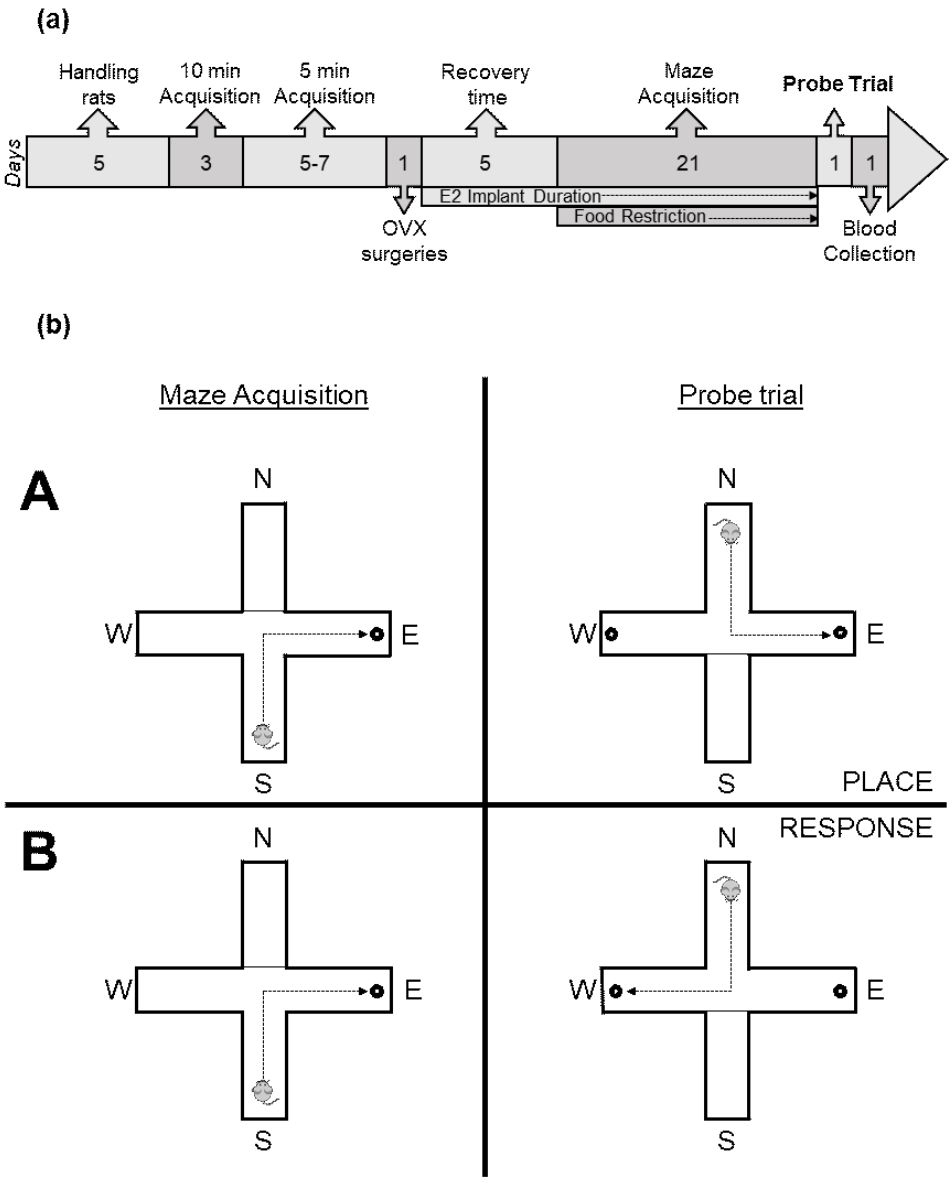

Figure 1. Maze training protocol and illustration of place and response memory in a dual-solution task. (a) Graphic outlining the 44-day maze training protocol beginning from initial handling on day 1 and ending with collection of blood samples on day 44. (b) Illustration of how place and response navigation are dissociated in a dual-solution task. In panel A, the rat is consistently trained to turn right, and on the probe trial turns left towards the same spatial location — indicating it used place memory. Panel A illustrates place memory being used. In panel B, the rat is trained to consistently turn right, and on the probe trial turns right, the same direction it had habitually been trained to turn indicating it used response memory.

Hormone Assays. 24 hours following the final hormone injection blood samples were collected and immediately stored on ice before being centrifuged, no anticoagulants were used. Serum was isolated and stored at -20˚C. Serum E2 and P concentrations were analyzed via

competitive enzyme-linked immunosorbent assays (ELISA; Enzo Life Sciences). For E2, the 17β-estradiol high sensitivity ELISA kit (Enzo Life Sciences; ADI-901-174) was used. This kit reports a sensitivity of 14.0pg/ml (range 15.6-1,000pg.ml). Intra-assay variability for E2: 68.2pg/mL (+/- 10.3); Inter-assay variability for E2: low E2, mean = 11pg/mL (+/- 0.3), 2.72 %CV; high E2, mean = 43.5pg/mL, (+/- 0.1), 0.23 %CV. For P, the progesterone high sensitivity ELISA kit (Enzo Life Sciences; ADI-900-011). This kit reports a sensitivity of 8.57pg/mL (range 15.62-500pg/ml). Intra-assay variability for P, mean = 134.26 (+/- 10.6); Inter-assay variability for P, mean = 94.9 (+/- 0.3), 0.32 %CV.

Statistical Analysis. All statistical analyses were performed using Microsoft Excel with the Analysis ToolPak for statistical analysis add-on. An analysis of variance (ANOVA) was carried out to compare groups for days to reach criterion. This experiment used a between-subjects design with categorical data and thus, nonparametric statistics were used. Five independent 2x2 chi-square tests were conducted to determine whether any treatment condition showed a significant bias toward place or response relative to what would be expected by chance i.e., 50/50. Finally, an odds ratio for each of these analyses was also calculated in lieu of effect sizes to determine if there was an association between treatment condition and the likelihood of using either place or response memory.

Results

There were no statistically significant differences in the number of days it took for rats to reach criterion between treatment conditions F (4,115) = 1.78, p = 0.137 (see Fig. 2a). Only those rats who received E2+P (1h) showed a statistically significant bias towards using a response strategy $\chi 2$ (1,23) = 4.17, p = 0.04 (Fig. 2b). No other treatment condition showed a statistically significant bias towards using place or response memory. Odds ratios show that rats treated with high E2 were 2.78x more likely to use place memory compared to those treated

with low E2. Whereas those treated with high E2+P (1h) were 4.05x more likely to use response memory compared to those treated with high E2 alone. Those treated with high E2+P (4h) were 2.78x more likely to use response memory compared to those given high E2 alone. Serum levels of E2 and P can be found in Table 1. 24h following the final injection, E2 and P were at their expected levels according to Almey et al. (2013). Four outliers were removed from the final calculation as they were more than 2 standard deviations outside the mean.

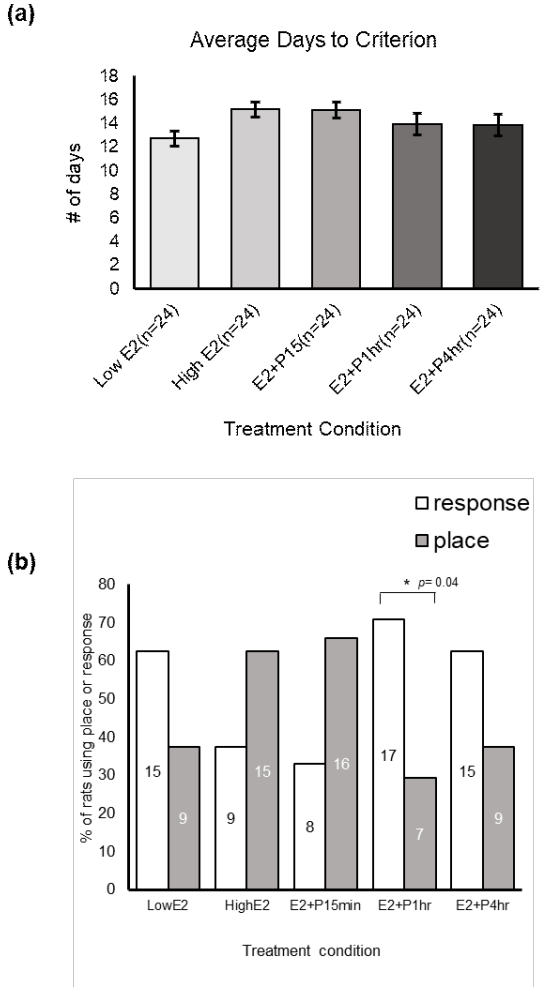

Figure 2. Use of place memory versus response memory in female rats treated with low E2, high E2, high E2+P at three different time points (i.e., 15 minutes, 1 hour, 4 hours). Numbers in bars represent actual number of animals. Significant bias to use response memory was observed in the high E2+P (1 hour) condition. E2, 17β-estradiol; P, progesterone.

Table 1. *Serum hormone levels (pg/mL)*

Hormone Condition	E2 levels (+/- SEM)	P levels (+/- SEM)
E2 capsule only (*n*=22)	35.5 (2.5)	-
E2 capsule + E2 (*n*=17)	51.85 (7.4)	-
E2 capsule + E2 + P (*n*=16)	52.32 (5.5)	94.9 (0.3)

**Serum was collected 24 hours following the last hormone injection.*

Discussion

Consistent with previous reports (Almey et al., 2014; Hussain et al., 2013; Quinlan et al., 2013, 2008), we have demonstrated that ovariectomized female rats replaced with low E2 predominantly use response memory, and that those replaced with high E2 predominantly use place memory in the dual-solution plus-maze task. When rats were treated with both P and high E2, they reverted to using response memory. However, this shift to response memory only occurred when P was administered 1h or 4h before testing. Rats administered P 15 min before testing showed a place memory bias like that of rats treated with high E2 alone. We suspect that 15 min may not have been enough time for a s.c. injection of P to reach the brain. When P was given 1h or 4h before testing, rats then showed a response memory bias. This is consistent with Korol and Pisani (2015) who showed that administration of P reversed the place memory promoting effect of EB administered alone. Thus, E2 promotes hippocampus-mediated place memory, and P rapidly dampens this effect and promotes the use of DS-mediated response memory.

Our findings that E2 promotes while P impairs hippocampus-mediated memory has been demonstrated by several others. For example, Chesler and Juraska (2000) administered female rats either E2 (5μg) or P (500μg) alone or together and showed deficits in latency in a MWM only when both E2 and P were administered together and not when either was given alone. Similarly, female rats in proestrus (characterized by elevated levels of both E2 and P) show impairments in completion of the MWM. This impairment is not present in female rats during pro-estrus characterized by elevated E2 but low P (Warren and Juraska, 1997). Others have similarly shown a performance-enhancing effect of E2 and an impairing effects of P on hippocampus-mediated spatial tasks (Barros et al., 2015; Bimonte-Nelson et al., 2003, 2004a, 2006; Harburger et al., 2007).

Given that we observed a change in memory bias 1h after P was administered, we suspect that P could be acting non-genomically to impair hippocampal place memory. One hour is less time than would be expected if P were acting genomically, which involves protein synthesis (Garg et al., 2017; Gellersen et al., 2008). It is therefore possible that P is exerting the rapid effect we observed through non-classical P signaling pathways via progesterone receptors outside of the cell nucleus via membrane-associated classical PRs or G-protein coupled PRs (Frick and Kim, 2018a; Garg et al., 2017). When P binds to these membrane-associated progesterone receptors, it can rapidly alter intracellular signaling cascades and metabolic processes (Frick and Kim, 2018a).

Past reports have shown that the HPC is rich with G-protein coupled progesterone receptors, specifically mPRβ (Intlekofer and Petersen, 2011; Zuloaga et al., 2012) and mPRδ (Pang et al., 2013). Others have shown that mPRβ and mPRδ are also present within the caudate putamen (Pang et al., 2013). Thus, the HPC and the DS both have the capacity to be altered rapidly by P via non-classical progesterone action, possibly through binding at mPRβ or

mPRδ. However, whether the effect we observed was due to binding at genomic or non-genomic receptors was not directly tested in our study.

The scope of our study was limited as several other factors that we did not measure could be influencing our results. For example, our findings with P could be attributed to its metabolite, allopregnanolone (ALLO) which has been shown to act as an allosteric agonist for $GABA_A$ receptors (Majewska et al., 1986). $GABA_A$ receptors are abundant in the HPC (Palpagama et al., 2019), and inhibitory $GABA_A$ receptor activity alters hippocampal spatial memory (Dashniani et al., 2020; Lecourtier et al., 2011). Johansson et al., (2002) showed that administration of ALLO (2mg/kg) impaired MWM performance in female rats, but only when administered 8 min (not 20 min) before testing. However, the effects of ALLO in that study were acute and short lived, and therefore may not help explain the timing of the effects we observed with P.

Our results may also have been influenced by the rats used in our experiment being ovariectomized. Ovariectomy significantly alters serum hormone levels, monoamines, and other catabolic/anabolic enzymes in female rats (Long et al., 2018). In fact, the HPC is particularly sensitive to the neurochemical alterations that result from OVX, whereas the DS may not be (Long et al., 2018). Moreover, administering E2 to an ovariectomized rat increases 5-HT and dopamine levels in the striatum which could have impacted our findings (Long et al., 2019). Additionally, OVX and hormone-replacement also significantly impact brain energy metabolism (Kirshner et al., 2020) which could be driving some of the effects we observed (Prakapenka and Korol, 2021).

Our study was limited by our post-test hormone analysis. First, serum samples were all collected 24 hours after testing which limits our ability to know the level of circulating E2 and P levels at the time of testing. In addition, there is growing controversy related to using

commercial ELISA kits for measuring steroid hormones (Rosner et al., 2013; Schultheiss et al., 2018). LC/MS and GC/MS have been shown to be much more reliable and valid techniques when it comes to the quantification of steroid hormones, especially at low levels (Li et al., 2016; Schultheiss et al., 2018).

Conclusion.

Our findings indicate that the regulation of place and response memory during spatial navigation is sensitive to changes in ovarian hormones. Specifically, we have demonstrated the importance of considering more than just E2 in the context of memory bias and spatial navigation in female rats. It is clear that E2 rarely acts in isolation and a more nuanced analysis which includes P, its metabolites, and other neurochemical endpoints is merited in future studies on this topic. Finally, we have demonstrated that P exerts its effects rapidly, which may result from P binding at non-classical receptors in the HPC and the DS.

Preface to Chapter 2.

In chapter 1 it was established that endogenous hormones like E2 and P had an impact on memory bias during spatial navigation. The next aim of this thesis was to investigate how exogenous hormones, such as those used in hormonal contraceptives might impact memory

bias in female rats. However, first an animal model of human hormonal contraception with appropriate ecological validity had to be developed. Therefore, in chapter 2 previous studies on modelling hormonal contraceptives in female rats were thoroughly reviewed. In addition, the development of our own model of hormonal contraception in female rats is outlined.

Chapter 2: Modeling hormonal contraception in female rats: a framework for studies in behavioral neurobiology.

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Abstract

Research on hormonal contraceptives (HC) in animal models is lacking, and as a result, so is our understanding of the impact of HC on the brain and behavior. Here, we provide a review of the pharmacology of HC, as well as the methodology and best practices for designing a model of HC in female rats. We outline specific methodological considerations regarding dosing, route of

administration, exposure time/timing, and selecting a control group. We also provide a framework outlining important levels of analysis for thinking about the impact of HC on behavioral and neurobiological outcomes. The purpose of this review is to equip researchers with foundational knowledge, and some basic elements of experimental design for future studies investigating the impact of HC on the brain and behavior of female rats.

Keywords: Hormonal contraceptives, animal models, ethinyl estradiol, progestin, levonorgestrel, methodology, female rats, dose.

Introduction

Over 150 million women use hormonal contraceptives (HC) globally, and this number is growing (United Nations, 2019). Despite the widespread use of HC, we understand very little about their effects on the brain and behavior. A fundamental reason for this lack of understanding is that experiments in animals are limited. Researchers interested in studying HC in animal models may have questions about how to design an animal model of HC. Thus, the

purpose of this review is to outline important considerations when designing experimental models of HC, specifically in female rats.

Here, we outline methodological considerations for the selection of dose, route of administration (ROA), exposure time, and selection of control groups as it relates to studying the effects of HC in female rats. Moreover, we provide a framework for designing experiments in rats with the intention of translating findings from studies in humans. The guidelines provided here are aimed to help navigate the sometimes complicated methodology of research on HC.

Pharmacology of HC.

How HC work. In order to understand the primary mechanism of action of HC, it is important to first describe the human menstrual cycle. The menstrual cycle is approximately 28 days long but can range anywhere between 21 to 35 days in length (Stanczyk et al., 2013). The 28-day cycle is divided into three broad phases: follicular, ovulatory, and luteal. Some separate the follicular phase into early-follicular (menses) and late-follicular, which are broadly separated by either low or high levels of 17β-estradiol (E2), respectively (Hampson, 2020). Within each of these phases, there are also distinctive changes to ovarian follicles (Grive, 2020; Grive and Freiman, 2015), the endometrial lining of the uterus (Hawkins and Matzuk, 2008), and in circulating levels of gonadal hormones (Hampson, 2020).

The menstrual cycle is regulated by signaling between the hypothalamus, the pituitary gland, and the ovaries a.k.a. the hypothalamic-pituitary-gonadal (HPG) axis. Declining levels of E2 and progesterone (P) in the late-luteal phase act as a negative-feedback signal to the hypothalamus. As a result, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) to the anterior pituitary to stimulate the release of the gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This marks the start of the early follicular phase, whereby FSH promotes follicle growth. By the second week of the follicular phase, a dominant follicle continues to proliferate in size while secreting increasing concentrations of E2. Around cycle days 12-13, these elevated levels of E2 prompt a surge of LH (Mesen and Young, 2015;

Sugino, 2014). This LH surge promotes further secretion of E2 which typically induces ovulation within ~24-36 hours (Stanczyk et al., 2013; Sundström Poromaa and Gingnell, 2014).

After ovulation, levels of E2 decline, and the dominant follicle undergoes luteinization forming the corpus luteum (Saltzman et al., 2011). The corpus luteum acts as a temporary endocrine gland, secreting primarily P, and to a lesser degree, E2 (Carr et al., 1982). The corpus luteum continues to proliferate for ~14-days, all the while secreting P and E2. If fertilization of the ovum occurs, pregnancy is initiated, and the corpus luteum begins to secrete substantial levels of P and E2 (Stanczyk et al., 2013). However, if the ovum is not fertilized, the corpus luteum regresses resulting in a decline in circulating ovarian hormones. This reduction in circulating ovarian hormones acts as a signal to the hypothalamus to recommence the cycle.

Most HC contain potent synthetic analogs of E2 and/or P which act directly at the level of both hypothalamus and pituitary. As a result of their binding, GnRH is suppressed which results in a subsequent decline in LH and FSH (Rivera et al., 1999). Without the gonadotropins, maturation of the ovarian follicle is suppressed, and ovarian hormone production is significantly diminished. As a result, there is no increase in circulating E2 to prompt the LH surge leading to the release of an oocyte. Thus, a state of acyclicity is achieved and ovulation does not occur. In women taking HC, circulating levels of pituitary and ovarian hormones (e.g., LH, FSH, E2, and P) typically decline to levels lower than what would be observed in the follicular phase in naturally cycling women (Montoya and Bos, 2017; Stanczyk et al., 2013). It should also be noted that a second mechanism by which HC work is by altering chemical composition of cervical mucus (Bagros, 1979; Han et al., 2017) and inhibiting the uterine cycle and endometrial growth (Dickey, 2021; Rivera et al., 1999).

Estrogens used in HC.

Past forms of HC have contained mestranol. Mestranol, the 3-methyl ether of ethinyl estradiol (EE), is a pro-drug for EE that is demethylated in the liver to its active form. Mestranol is no longer used in HC (Fotherby, 1996; Hampson, 2020). Some newer HC use estradiol

valerate (EV), a pro-drug for the endogenous E2 therefore considered to be bioidentical to E2 (Cirigliano, 2007). EV itself is not very bioavailable (Table 1) and is far less potent than E2. Yet, once it is hydrolyzed to E2, its pharmacological properties are similar to those of endogenous E2 (Kuhl, 2005).

The vast majority of HC contain the synthetic estrogen EE (Hampson, 2020). EE differs from endogenous E2 in several ways. For example, circulating E2 is metabolized by 17β-hydroxysteroid dehydrogenase (17β-HSD) which converts E2 to estrone (E1). E1 has only ~4% of the binding affinity of E2 for estrogen receptors (ER; Kuhl, 2005). Unlike E2, EE is not metabolized by 17β-HSD (Fotherby, 1996) and thus, EE remains in circulation longer and continues exerting its potent estrogenic effects (Table 1). EE has a terminal half-life of 10 to 20h, but when dosing repeatedly at 24h intervals, EE bioaccumulation is 23-77% (Stanczyk et al., 2013). Unlike E2, EE does not bind to sex hormone-binding globulin (SHBG), therefore 98.5% of EE is bound to serum albumin (Kuhl, 2005), leaving only ~1% as free circulating EE. EE also induces a significant rise in hepatic production of hormone-binding globulins, like SHBG and corticosterone-binding globulin (CBG; Kuhl, 2005; Wiegratz et al., 2003). Lastly, EE has a 17α-ethinyl group that can be oxidized. If this occurs, it results in a highly reactive intermediate that can irreversibly inhibit CYP enzymes that are important for steroid metabolism (Kuhl, 2005).

EE has twice the binding affinity of E2 for estrogen receptor α (ERα; Table 1). There have been mixed reports on its binding affinity for estrogen receptor β (ERβ; (Escande et al., 2006; Jeyakumar et al., 2011) and its affinity for the membrane-bound estrogen receptor g protein-coupled estrogen receptor 1 (GPER) has not been reported. Nevertheless, EE does have effects on GPER binding that are not observed in GPER knock-out mice (Yates et al., 2010). Dickson and Eisenfeld (1981) compared EE and E2 on their ability to promote nuclear translocation for the hepatic ER. Approximately 100x the concentration of E2 was required to produce the same receptor translocation observed with EE (Dickson and Eisenfeld, 1981). The

important takeaway is that EE produces greater estrogenic effects and remains in circulation longer and at higher concentrations than endogenous E2.

Table 1) Summary of the pharmacology of three types of estrogens used in hormonal contraceptives.

Types of estrogen	Pharmacokinetics			Pharmacodynamics*	
	C_{max} / T_{max}	Bioavailability	Half Life	ERα (RBA%)	ERβ (RBA%)
Ethinyl estradiol (EE)	30µg EE (oral) = C_{max}90-130pg/mL / T_{max}1-2h [(I)] 35µg EE (oral) C_{max}174±67pg/mL / T_{max}1.3h±0.5 [(II)]	Oral: 45% [(I)]; 38-48% [(IV)]; 55% [(V)] Vaginal: 55% [(IV)]	5-30h [(I)]; 7h [(V)]	233±41 [(VIII)]; 194±22 [(IX)] Agonist	37.8±5.1 [(VIII)]; 151±27 [(IX)] Agonist
Mestranol (MES)	**Measured as serum EE* 50µg MES (oral) C_{max}175±72pg/mL / T_{max}1.9h±0.8 [(II)]	MES is orally inactive. 50µg MES is bioequivalent to 35µg of EE [(II)]	50 min [(VII)]	Unspecified ER 2.262 [(X)] * Agonist	
Estradiol valerate (EV)	**Measured as serum E2* 2mg EV (oral) C_{max}90-140pg/mL / T_{max}1-3h [(III)]	Oral: 5% [(IV)] 2mg EV (oral) = 3% (as E2)	50min [(I)]; 90 min [(VI)]	100 Agonist	

Note. References: (I) Stanczyk et al., 2013; (II) Goldzieher and Brody, 1990; (III) Düsterberg and Nishino, 1982; (IV) Kuhl, 2005; (V) Fotherby, 1996; (VI) von Schoultz et al., 1989; (VII) Rabe et al., 2000; (VIII) Escande et al., 2006; (IX) Jeyakumar et al., 2011; (X) Blair, 2000.

Progestins used in HC.

Synthetic analogs of P are referred to as *progestins* (Stanczyk, 2003). Some progestins are given orally as prodrugs which must first be metabolized before exerting their contraceptive effects. For example, norethynodrel is metabolized to norethisterone (NET), desogestrel (DSG) to 3-keto-desogestrel, and norgestimate (NOR) to levonorgestrel (LNG; Schindler, 2015). Orally administered progestins usually reach their peak serum concentration by ~1-2h (Table 2). Progestins administered orally generally have good bioavailability in humans (>60%; Table 2). The elimination half-life for progestins administered orally ranges from 8h to ~30h (Table 2). However, progestins cannot all be thought of as a homogeneous group of molecules (Giatti et al., 2016; Stanczyk, 2003). Progestins are often classified either according to their generation (Petitti, 2003) or to their chemical structure and physiological effects (Giatti et al., 2016; Sitruk-Ware, 2008; Stanczyk, 2003).

Table 2) *Summary of progestins used in hormonal contraceptives and their pharmacology.*

Types of Progestins		Pharmacokinetics			Pharmaco dynamics*	
	Progestin Name	C_{max} / T_{max}	Bioavailability	Half Life	PR	AR

		(*abbrev.*)				(RBA%)	(RBA%)
Androgenic	***19-nortestosterone*** *Estranes*	**Norethynodrel** *active as* ***Norethisterone*** (NET) a.k.a. norethindrone	500µg NET= C_{max}6ng/ml 300µg NET= C_{max}4ng/ml / T_{max}1-2h 1mg NET+120µg EE= C_{max}16ng/ml	47-73% & 1000µg NET 64% ± 8%	1mg NET 50µg + EE (intravenous) 8h ± 3.3h (5.2-12.8h)	75 Agonist	15 Agonist
		Norethisterone Acetate (NETA)	**Measured as serum NET* 0.5mg NETA (oral) = C_{max}5ng/ml / T_{max}1h 1mg NETA (oral)= C_{max}5ng/ml 2mg NETA (oral)= C_{max}12ng/ml 1mg NETA+2mg E2 (oral)=C_{max}8.5ng/ml / T_{max}1h	*Rapidly converted to NET*	7.6h	75 Agonist	15 Agonist
	19-nortestosterone *Gonanes*	**Levonorgestrel** (LNG) similar to norgestrel or NG	250µg LNG (oral) = C_{max}6.0ng/ml 50µg LNG+30µg EE= C_{max}2.0ng/ml / T_{max}1h 75µg LNG+50µg EE (oral)= C_{max}2.5ng/ml / T_{max}1-3h 100µg LNG+20µg EE=C_{max}2.4ng/ml / T_{max}1h 125µg LNG+30µg EE=C_{max}4.3ng/ml / T_{max}1h 150µg LNG+30µg EE (oral)=C_{max}3.5/4.3ng/ml	250µg LNG (oral) 99% ± 20% (72-125%) 150µg LNG (oral) 89% ± 13% (65-108%)	250µg LNG (oral) 9.9 ± 0.7h 150µg LNG (oral) 13.2 ± 6.0h	150 Agonist	45 Agonist
		Norgestimate (NOR) **Note:** *NOR is metabolized to deacetylated NOR* a.k.a. **Norelgestromin (**NGM**)**	**Measured as serum NGM* 360µg NOR+70µg EE (oral) = C_{max}4ng/ml / T_{max}1h **Measured as serum LNG-3-oxiome* 250µg NOR+35µg EE (oral) = C_{max}2.5ng/ml /T_{max}1.5h	250µg NOR+35µg EE (oral) *22% ± 6% of NOR dose becomes systemically available as LNG	360µg NOR+70µg EE (oral) **NGM elevated in serum after* >36h.	15 Agonist Activator	0 Partial Agonist
		Desogestrel (DSG) *active as 3-ketodesogestrel* a.k.a. ethonogestrel or ENG	150µg DSG+30µg EE=C_{max}3.69 ± 0/97ng/ml /T_{max}1.6 ± 0.97h	150µg DSG and 30µg EE (IV) 76% ± 22% (oral) 62% ± 7%	150µg DSG and 30µg EE 11.9 ± 4.1h & 23.8 ± 5.3h & 11.2h	150 Agonist	20 Agonist
		Gestodene (GTD)	50µg GTD+30µg EE =C_{max}1.0ng/ml 75µg GTD+30µg EE=C_{max}3.6ng/ml 100µg GTD+30µg EE=C_{max}7.0ng/ml/T_{max}1.4–1.9h	75µg GTD 99% ± 11% (86-111%) & 87% ± 19% (64-126%)	75µg GTD 12h-14h 11.2h	90 Agonist	85 Agonist
Anti-androgenic	*19-nor testosterone*	**Dienogest** (DNG)	1mg DNG= C_{max}23.4ng/ml T_{max}2.2h ± 1.1h 2mg DNG+30µg EE= C_{max}53ng/ml / T_{max}2h	1mg DNG (oral) 95%-96.2%	6.5h 9.1h	5 Agonist	10 Antagonist
	17-OH progesterone	**Medroxyprogesterone*** (MPA)	10mg MPA (oral)= C_{max}3-5ng/ml / T_{max}1-4h	<15% & 100%	10mg MPA (oral) = 24h	115 Agonist	6 Agonist
		Cyproterone acetate (CPA)	2mg CPA + 35µg EE = C_{max} 15.2ng/ml / T_{max}1.6h 1mg CPA = C_{max} 11ng/ml	68-100%	1.6 to 4.3 days (oral)	90 Agonist	6 Competitive Antagonist
		Chlormadinone acetate (CMA)	2mg CMA + 30ug EE (oral) = C_{max} 1.6ng/ml / T_{max}1-2h	100%	25-89h	67 Agonist	5 Antagonist
	19-nor progesterone	**Nomegesterol acetate** (NOMAc)	5mg NOMAc (oral) = C_{max} 8ng/ml / T_{max} 4h	63%	35-50h	125 Agonist	42 Antagonist
		Segesterone Acetate a.k.a. **Nestorone** (NST)	100µg NST (oral)= C_{max}160pg/ml / T_{max}n/a	100µg NST (oral) 10%	100µg NST (oral) 1-2h	136 Agonist Activator	0 Agonist
	spironolactone	**Drospirenone** (DRSP)	3mg DRSP+30µg EE (oral) = C_{max}36.9ng/ml 3mg DRSP (oral) = C_{max}35ng/ml / T_{max}1-2h	1-10mg DRSP (oral) 66% & 76-85%	3mg DRSP+30µg EE (oral) 31.1 to 32.5h & 1.6-27h	35 Agonist	65 Inverse Agonist

Note. *MPA is categorized with anti-androgenic progestins, but it can have androgenic effects. **References**: ***Pharmacokinetics****:* (a) Kuhl, 2005; (b) Stanczyk, 2003; (c) Kuhnz et al., 1993; (d) Ruan et al., 2012; (e) Fotherby, 1996; (f) Oettel et al., 1999; (g) Barradell and Faulds, 1994. ***Pharmacodynamics****:* The values for RBA% for AR and PR were retrieved from Kuhl (2005) & Schindler et al., (2008) and are compiled from cross-comparisons. Results are from in vitro experiments which vary on incubation conditions a biological tissue tested, thus, these values are not standardized to a single species or tissue type. RBA% is reported relative to: PR: Progesterone receptor (promegestone = 100%), AR: Androgen receptor (metribolone R1881 = 100%). *All agonist / antagonist information was retrieved from DrugBank (Wishart et al., 2018).

Classifying progestins by generation. Progestins are sometimes classified according to the generation they were released publicly as contraceptives (Petitti, 2003). However, others classify progestin generations according to when the progestin was first synthesized and their molecular structure (Giatti et al., 2016; Sitruk-Ware, 2008; Stanczyk, 2003). As a result, the

classification of progestins by generation in the literature is often inconsistent. For example, medroxyprogesterone acetate (MPA) and cyproterone acetate (CPA) are both derived from 17-OH progesterone (Sitruk-Ware, 2008) and are both sometimes classified as first-generation progestins (Davtyan, 2012). However, MPA is an agonist for androgen receptors (AR), whereas CPA is a competitive antagonist for AR (Barradell and Faulds, 1994; Bentel et al., 1999; Neumann, 2009). Moreover, unlike MPA and CPA (derived from 17-OH progesterone), another progestin also classified as first-generation, norethisterone (NET; Davtyan, 2012; Tricotel et al., 2015) is actually derived from 19-nor testosterone. Even classifying NET as a first-generation is inconsistent with other authors who classify NET as a second-generation progestin along with others derived from 19-nor testosterone (Giatti et al., 2016; Stanczyk, 2003).

Another illustration that classification by generation is problematic is the classification of norgestimate (NOR) as a third-generation progestin (Anand et al., 2015; Davtyan, 2012; Tricotel et al., 2015). NOR is rapidly metabolized to form 17-desacetylnorgestimate, which can later be metabolized to LNG (Ahire et al., 2017; Kuhl, 2005; McGuire et al., 1990). LNG is a progestin most often classified as second-generation, even by the same authors (Anand et al., 2015; Davtyan, 2012; Tricotel et al., 2015). Thus, NOR (third-generation) once metabolized becomes predominantly active as LNG (second-generation). This calls into question the utility of classifying NOR as a third-generation rather than second-generation like LNG, or the utility of classifying progestins by generation at all.

Fourth-generation progestins are broadly defined as 1) being the newest formulations of HC and 2) having anti-androgenic effects. However, the progestins classified as fourth-generation are not structurally similar. For example, nestorone (NST) is a 19-nor progesterone derivative, and drospirenone (DRSP) is a derivative of spironolactone (Sitruk-Ware, 2008, 2005; Sitruk-Ware et al., 2013) and both have dramatically different binding affinities for AR (Kuhl, 2005). Dienogest (DNG) is also classified as a fourth-generation progestin due to its anti-androgenic effects, despite being a derivative of 19-nor testosterone which are usually classified

in generations 1 through 3 (Giatti et al., 2016). The essential element, therefore, is not to consider the progestin generation, but whether it exerts behavioral or neurobiological effects that are more similar to those of endogenous P or of the androgens. This distinction is based on the pharmacodynamics and the physiological effects of the progestin and is likely more useful than a broad classification of progestins by generation.

Androgenic vs. anti-androgenic effects of progestins. Another way to think about progestins is whether they exert androgenic or anti-androgenic effects. Progestins each have different binding affinities for AR and progesterone receptors (PR; Table 2). Thus, some progestins exert physiological effects that are more typical of androgens, while others may exert effects more similar to those of P (Sitruk-Ware, 2008). Progestins structurally related to testosterone (T) are NET, norethisterone acetate (NETA), NOR, norgestrel (NG), LNG, DSG, and gestodene (GTD; Table 2). Given their structural similarity to T and affinities for AR these progestins exert androgenic effects (Giatti et al., 2016; Stanczyk, 2003). However, progestins such as LNG and NET can also simultaneously produce physiological effects that are anti-androgenic. For example, LNG and NET both induce a significant decrease in serum levels of free T and inhibit the activity of 5α-reductase which synthesizes the highly androgenic dihydrotestosterone (DHT) from T (Stanczyk, 2003). Moreover, several other progestins that would otherwise be classified as “androgenic” such as MPA, DSG, or NOR can have subtle anti-androgenic effects via weak agonism of ERα (Kuhl, 2005; Wishart et al., 2018).

Progestins that are structurally related to P are NST, nomegestrol acetate (NOMAc), CPA, chlormadinone acetate (CMA), and MPA (Table 2). These progestins exert anti-androgenic or progestogenic effects but have less structural homogeneity than androgenic progestins. For example, MPA and CPA are both derived from 17-OH progesterone and therefore share a similar structure to P. However, CPA is the most potent anti-androgenic progestin (Barradell and Faulds, 1994; Neumann, 2009) whereas MPA can have androgenic effects (Bentel et al., 1999; Kuhl, 2005). Unlike MPA and CPA, DNG is structurally related to

testosterone, yet, unlike the other 19-nor testosterone derivatives, it has no androgenic effects and is anti-androgenic through antagonism of AR (Oettel et al., 1999). In addition, the derivative of spironolactone, DRSP, also exerts anti-androgenic effects but via inverse agonism of AR (Elger et al., 2003; Muhn et al., 1995). Thus, while several progestins have anti-androgenic effects, they do so through different mechanisms.

Additionally, looking at relative binding affinity (RBA) alone can be misleading for comparing progestins one to one. For example, DRSP has an RBA for AR similar to that of LNG or GTD (Table 2). However, DRSP is an inverse agonist to AR, while LNG and GTD are full agonists to AR. Thus, comparing progestins based simply on RBA can be misleading given that they could exert opposite effects despite having a similar RBA for AR or PR. It should also be noted that many progestins are "dirty drugs" and bind to several other targets besides AR and PR. For example, most progestins listed in Table 2 also have binding affinities for glucocorticoid and mineralocorticoid receptors (see Kuhl, 2005).

Given that RBA alone doesn't accurately reflect how any particular progestin may impact physiology or behavior, progestins can also be understood in terms of their so-called "*androgenicity*". The androgenicity of a progestin refers to the degree to which a progestin exerts effects that are typical of endogenous or synthetic androgens such as T and DHT, or metribolone. Some progestins have higher androgenicity such as LNG and NETA. While others have low androgenicity, for example, DRSP, DNG, CPA. Yet, how each progestin achieves its level of androgenicity is unique. Each progestin has some degree of androgenic activity and some degree of progestational activity (see Dickey, 2021; Table 4). The ratio between androgenic activity and progestational activity provides better insight into how a progestin may impact physiology and/or behavior. For example, LNG and GTD have similar levels of androgenic activity. Yet, GTD has more than twice the progestational activity of LNG (Dickey, 2021). Thus, the androgenic activity that would be observed with GTD is lessened due to its

simultaneous progestational activity. The androgenic/progestational activity ratio is therefore greater for LNG, and as a result, LNG has greater androgenicity compared to GTD.

What is clear is that there is no perfect way to cluster progestins into groups. While it is possible to loosely group them according to their physiological effects i.e., androgenic vs. anti-androgenic, this approach does not necessarily reflect similarities among the progestins structurally or mechanistically. In any case, broadly classifying progestins together (either by generation or by structure) will eventually lead to issues of reproducibility and consistency in the literature. The best approach moving forward, both in studies of humans and rodents, would be to study individual formulations with a specified progestin. Only by studying individual progestins will we be able to decipher the unique impact of any given type of HC on the brain and behavior.

Past studies on HC in female rats.

Table 3 contains a comprehensive list of studies that have examined the effect of HC on the brain and/or behavior in female rats. Forty-six studies were published between 1970 and 2022. Table 3 also includes studies that were not conducted in female rats but are nevertheless relevant studies for modelling HC in rodents. The earliest studies on HC (between 1970-1976) used estrogens such as mestranol and progestins like norethynodrel and lynestrol that are no longer used in HC. Most studies used EE as their estrogen component, although four studies used EV (Genazzani et al., 2007; Lenzi et al., 2008, 2009; Pluchino et al., 2009).

There were seven experiments between 1976 and 1998 which examined progestins such as NOR, NET, and NETA (Baker et al., 1977; Daabees et al., 1981; Genazzani et al., 1989; Jori and Dolfini, 1976; Ladinsky et al., 1976; Picazo et al., 1998; Srivastava and Laumas, 1975; Tejwani et al., 1985). After 2002 most experiments studying HC in female rats used LNG as their progestin. Two studies examined the impact of DRSP but tested ovariectomized (OVX) female rats (Genazzani et al., 2007; Koebele et al., 2022). Other studies have examined the impact of MPA or LNG on the brain and behavior in female rats. However, these were conducted in the context of surgical menopause using OVX middle-aged rats and were

therefore not included in Table 3 (Braden et al., 2017, 2011, 2010; Chisholm and Juraska, 2012; Koebele et al., 2021, 2017; Lowry et al., 2010; Prakapenka et al., 2018). To our knowledge, no experiments examining the brain and/or behavior in gonadally-intact female rats have tested the following progestins used in HC: CMA, CPA, DNG, DSG, GTD, NOMAc, and NST.

The majority of previous studies on HC (63%) used gonadally-intact female rats. There was no predominance of any rat strain used, although Wistar and Sprague-Dawley rats were used often. Over half of the studies (65%) examined both EE combined with a progestin in their model. Roughly 13% studied EE administered alone and 17% gave a progestin only. Doses of EE ranged from 0.2μg/kg (Nwakanma et al., 2021) to 100μg/kg (Baker et al., 1977) and doses of LNG ranged from 0.43μg/kg (Nwakanma et al., 2021) to 500μg/kg (Graham and Milad, 2013). In most studies, HC were administered to rats using oral gavage (28.2%) or by subcutaneous injection (50%). Rats were administered HC as a single acute injection or chronically over ~4-60 days. The longest exposure time reported for HC administration was ~8 months (Fregly, 1972). However, the majority of the studies (61%) administered HC between 20 and 60 days (Table 3).

Slightly less than half (48%) of the studies examined the impact of HC on vaginal cytology. Only 7 studies (15.2%) measured the impact of HC on serum gonadotropin or ovarian hormone levels (Allaway et al., 2021; Follesa et al., 2002; Graham and Milad, 2013; Hilz et al., 2021; Porcu et al., 2012; Sassoè-Pognetto et al., 2007; Simone et al., 2015). Of these 7 studies, two of them did not report the actual hormone values, only the relative % change from controls (Graham and Milad, 2013; Simone et al., 2015). Only 5 studies compared rats treated with HC to naturally cycling female rats at different stages of the estrous cycle (11%), while most (89%) compared them to vehicle / sham treatment without regard to cycle phase. From 1970 to 1998, research on HC in female rats did not examine behavior outside of some simple tests of locomotor activity (Baker et al., 1977; Fregly, 1973, 1972; Fregly et al., 1970). However, since ~2002 HC have shown mixed effects (or no effect) on a wide range of behavioral tasks and biomarkers (see Table 3 for summary).

Table 3) ***Summary of past studies examining the effect of hormonal contraceptives on the brain and behavior in female rats.***

Publication (by year)	Intact ?	Rat strain	Dose(s)	Route(s) of Administration	Exposure Time(s)	Confirmed Acyclicity?	Control group(s)	Behavioral Task(s)	Bioma
Lacasse et al., 2022b	Intact	Long-Evans	EE = 10µg/kg LNG = 20µg/kg	Subcutaneous injection in 5% ethanol/sesame oil; vol. ~0.2mL	Daily for 21 days	YES *Vaginal Cytology* *LH assay*	Diestrus females given vehicle injection (sesame oil; vol. ~0.2mL)	DSPM Δ	LH Δ Body weight Δ
Boi et al., 2022	Intact	Sprague-Dawley	EE = 20µg LNG = 60µg	Oral gavage	Daily for 4 weeks	YES *Vaginal Cytology*	Vehicle (0.4% aqueous solution of sodium carboxymethyl cellulose	NOR ×; NPL ×; MWM ×	BDNF Δ; Histo methylation Δ; Phospho-CRE
Koebele et al. 2022 (study 1)	OVX	Fisher-344	DRSP = 12.5µg/ 30µg / 300µg	Subcutaneous injection in 0.1mL of sesame oil	Daily for 53 days	YES *Vaginal Cytology*	Vehicle (sesame oil)	WRAM Δ; MWM Δ; OFT ×	Body weight × weight ×; GAD65 & 67 e IGF1-R expres
Koebele et al. 2022 (study 2)	OVX	Fisher-344	EE = 0.125ug / 0.3ug DRSP = 30µg	Subcutaneous injection in 0.1mL of sesame oil	Daily for 53 days	YES *Vaginal Cytology*	Vehicle (sesame oil)	WRAM Δ ; MWM ×; OFT Δ	Body weight Δ weight Δ; GAD65 & 67 e IGF1-R expres

Nwakanma et al., 2021	Intact	Wistar	EE = 0.2µg/kg LNG = 0.43µg/kg	OC pill dissolved in distilled water and administered by syringe orally.	Daily for 21 / 42 / or 63 days	NO	Vehicle (water)	MWM Δ	Hippocampal: *histomorpholo* Hippocampal: specific enolas fibrillary acidic
Hilz et al., 2021	Intact	Sprague-Dawley	LNG = 30mg	Two 30mg Silastic tube implants into scapular region.	17 days	YES *Vaginal Cytology*	Proestrus and metestrus/diestrus female rats	CPP Δ; ultrasonic vocalizations Δ	VTA and SN: T Δ Hormones: E2 Uterine horn th
Maher et al., 2021	OVX	Long-Evans	EE = 0.3µg	Subcutaneous injection	Daily for 42 days	YES *Vaginal Cytology*	E2 group (3µg/0.1mL sesame oil) OVX (vehicle)	Nicotine demand and cue-induced seeking Δ	Body weight Δ
Wusu et al., 2021	Intact	Albino	EE = 0.03mg LNG = 0.015mg	Oral gavage	Daily for 21 days	NO	Vehicle (DMSO and distilled water)	*no data*	Brain, liver, an gene expressio catalase Δ; B-c Lymphoma 2 Δ Δ; caspase 3 Δ dismutase Δ
d'Adesky et al., 2021	Intact	Sprague-Dawley	EE= 0.03mg NG = 0.3mg	Oral gavage	Daily 16-21 days (3 days active 1 day placebo)	NO	Sham Gavage; Females receiving HC+Nicotine	*no data*	Histamine and metabolites Δ peptides ×
Allaway et al., 2021	Intact	Sprague-Dawley	ENG =0.30µg / 0.17µg / 0.09µg	Subcutaneously implanted slow-release pellet	29 days	YES *Vaginal Cytology* *Transabdominal ultrasound*	Vehicle	Food consumption ×	Body weight × volume Δ; follic E2 Δ.
Diaz and Raval, 2020	Intact	Sprague-Dawley	EE= 0.03mg NG = 0.3mg	Oral gavage	Daily 16-21 days (3 days active 1 day placebo)	NO	Sham Gavage; Females receiving HC+Nicotine	*no data*	Infarct volume mito CIV ×; gly TCA cycle met
Porcu et al. 2019	Intact	Sprague-Dawley	EE = 20µg LNG = 60µg	Oral gavage	Daily for 4 weeks	NO	Vehicle (1ml, 0.4% sodium carboxymethylcellulose)	*no data*	Plasma: Allo Δ corticosterone
Simone et al., 2015	Intact	Sprague-Dawley	EE = 10µg / 30µg LNG =20µg / 60µg	Subcutaneous injection in 5% ethanol / sesame oil	Daily for 21 days	YES *Vaginal Cytology*	Vehicle (metestrus/diestrus females with E2 levels <10.0pg/mL)	EPM Δ; SPDBΔ; OFT Δ; NOR Δ; NOP ×	E2 Δ, BDNF m Δ; tyrosine hyd mRNA Δ; galan mRNA/protein
Mennenga et al., 2015 (study 1)	OVX	Fischer-344	EE = 0.125µg / 0.3µg	Osmotic pump implant releases 0.15µl/h of 200ul across 6 weeks. *Tonic Administration*	Daily for ~60 days	YES *Vaginal Cytology*	Vehicle (propylene glycol)	WRAM Δ; MWM ×; Visual Platform task ×	Uterine Weigh
Mennenga et al., 2015 (study 2)	OVX	Fischer-344	EE = 0.125µg / 0.18µg / 0.3µg	Subcutaneous injections. *Cyclic Administration*	Daily for ~60 days	YES *Vaginal Cytology*	Vehicle (sesame oil)	WRAM Δ; MWM Δ; Visual Platform task ×	E1 Δ; E2 Δ; EE Basal Forebrai Δ
Santoru et al., 2014	Intact	Sprague-Dawley	EE = 0.03mg LNG = 0.125mg	Oral administration (unspecified method)	Daily for 4 weeks	NO	Vehicle (0.4% aqueous solution of sodium carboxymethylcellulose)	FST Δ; SPT ×; MWM ×; RIT Δ; PMT Δ	Cerebrocortica T Δ
Okojie and Oyekunle, 2014	Intact	Wistar	MPA = 13mg/1mL / 33mg/1mL	Intramuscular injection	Weekly for 3 weeks	NO	Vehicle (saline)	Y-maze Δ, locomotor activity Δ	*no data*
Graham and Milad, 2013	Intact	Sprague-Dawley	LNG = 100µg/kg / 500µg/kg	Subcutaneous injections of distilled water and dimethyl sulfoxide (2:1)	Daily for 4 days	YES *Vaginal Cytology*	Vehicle (distilled water) Proestrus and metestrus female rats	Fear Extinction Δ	E2 Δ
Olanrewaju et al., 2013	Intact	Wistar	MPA = 100mg/1mL or 200mg/1mL	Intramuscular injection	Weekly for 3 weeks	NO	Vehicle (saline)	Y-maze Δ, locomotor activity Δ	*no data*
Vega-Rivera et al., 2013	OVX	Wistar	EE = 1.25µg / 2.5µg / 5µg	Subcutaneous injection (dissolved in corn oil; vol. 0.2ml)	Single injection, 48h before test.	NO	Vehicle (corn oil; vol. 0.2ml)	FST Δ; locomotor activity ×	5-HT Δ; 5,7-DH DA Δ
Porcu et al., 2012	Intact	Sprague-Dawley	EE = 0.03mg LNG = 0.125mg	Subcutaneous injections (vol 1ml)	Daily for 4 weeks	NO	Vehicle	EPM Δ	Cerebrocortica plasma: Pregn Δ; Allo Δ; $GABA_A$ subunits Δ
Beck et al., 2012	OVX	Sprague-Dawley	MPA = 0.1mg/kg / 1.5 mg/kg	Subcutaneous injection	Daily for 4 days	NO	Vehicle (sesame oil)	Delay eyeblink conditioning Δ	*no data*
Raval et al., 2012	Intact	Sprague-Dawley	EE= 0.03mg NG = 0.3mg	Oral gavage	Daily 16-21 days (3 days active 1 day placebo)	NO	Sham Gavage; Females receiving HC+Nicotine	*no data*	Hippocampal r ERβ protein ×; activity ×; mito respiratio phosphorylate mito CIV subu ROS ×
Braden et al., 2011	Intact + OVX	Fischer-344	MPA = 3.5mg	Subcutaneous injections	Weekly for 4-8 months up to 13 months	YES *Vaginal Cytology*	Vehicle (sesame oil + DMSO)	WRAM Δ; MWM Δ; VPM ×	GAD65 & 67 × MPA Δ; body w uterine weight
Picazo et al., 2010	OVX	Wistar	EE = 1µg / 10µg / 100ug	Intra-peritoneal injection	Single injection	NO	Vehicle (cyclodextrin 0.1g/kg)	*no data*	Dentate gyrus: loss Δ; neural
Pluchino et al. 2009	OVX	Wistar	EV = 0.05mg/kg CMA = 0.1 / 0.5 / 1mg/kg	Subcutaneous injections	14 days	YES *Vaginal Cytology*	Vehicle (proestrus females and OVX females)	*no data*	Allo Δ; B-endo

Lenzi et al., 2009	OVX	Wistar	EV = 0.05mg/kg NST = 10µg / 50µg / 250µg/kg	Subcutaneous injections	14 days	YES *Vaginal Cytology*	Vehicle (proestrus females and OVX females)	*no data*	Allo Δ; B-endo
Lenzi et al. 2008	OVX	Wistar	EV = 0.05mg/kg NOMAc = 0.05 / 0.1 / 0.2 / 0.5 / 1mg/kg/day	Subcutaneous injections	14 days	YES *Vaginal Cytology*	Vehicle (proestrus females and OVX females)	*no data*	Allo Δ; B-endo
Genazzani et al. 2007	OVX	Wistar	EV = 0.05mg/kg DRSP = 0.1 / 0.5 / 1.0 mg/kg	Subcutaneous injections	14 days	YES *Vaginal Cytology*	Vehicle (proestrus females and OVX females)	*no data*	Allo Δ; B-endo
Sassoè-Pognetto et al., 2007	Intact	Sprague-Dawley	EE = 0.03mg LNG = 0.125mg	Subcutaneous injections (vol 1ml)	Daily for 4 weeks	NO	Vehicle Pregnant females	*no data*	Pregnanolone Allo Δ; gephyri protein ×; geph
Estrada-Camarena et al., 2004	OVX	Wistar	EE = 2.5µg / 5µg	Subcutaneous injection (vol. 0.2ml)	Single injection, 48h before test.	NO	E2 (2.5µg/5µg) Vehicle (corn oil)	FST Δ; OFT Δ	*no data*
Estrada-Camarena et al., 2003	OVX	Wistar	EE = 1.25µg / 2.5µg / 5µg/ 10 µg	Subcutaneous injection (vol. 0.2ml)	Single injection, 24h before test.	NO	E2 (2.5µg/5µg/10µg) Vehicle (corn oil)	FST Δ; OFT ×	*no data*
Follesa et al., 2002	OVX	Sprague-Dawley	EE = 0.03mg LNG = 0.125mg	Subcutaneous injections (in Tween 80 and distilled water; nape; vol 1ml)	Daily for 6 weeks	NO	Vehicle	EPM Δ	Pregnenolone Δ; GABA subunit expression Δ; receptor gene
Andrews et al., 2002	Intact	Wistar	EE = 0.01mg / 0.05mg / 0.2mg/kg	Oral Gavage	Daily >28days	YES *Vaginal Cytology*	Male rats	Locomotor activity ×; reflex ×	Body weight Δ organ weights strength ×; live Hormones: T4 T3 ×
Lemus et al., 1992 θ	GDX	Wistar (male rats)	LNG = 300µg EE = 5µg	Subcutaneous injection (vol. 0.1-0.2ml)	Daily for 21 days	*NA*	Vehicle (10% ethanol + Corn Oil). DHT control (300µg) +E2 (5µg)	Male sexual behavior Δ	Ventral prostat seminal vesicl
Picazo et al., 1998	OVX	Wistar	NET = 0.25 / 0.5 / 1mg LNG = 0.25 / 0.5 / 1mg	Subcutaneous injection (vol. 0.2ml)	Single injection 4h before test.	NO	Vehicle (dicholoromethane, corn oil)	SPDB Δ; ambulatory behavior Δ	*no data*
Genazzani et al. 1989	OVX	Sprague-Dawley	EB = 2mg NOR = 10µg / 100µg	Subcutaneous injections	14 days	NO	Vehicle (sham and OVX)	*no data*	B-endorphin Δ
Tejwani et al., 1985	Intact	Sprague-Dawley	EE = 1µg / 10µg NET = 15µg / 150µg	Oral gavage	Acute (5 days) Chronic (7 weeks)	NO	Vehicle (0.25ml of corn oil daily by gavage)	*no data*	B-endorphinΔ; met-enkephali enkephalin Δ
Daabees et al., 1981	Intact	Albino	EE = 0.05mg MPA = 12.5mg NETA = 1mg	Intramuscular injection (MPA) Oral treatment (EE and NEA; suspended in 0.5% carboxymethylcellulose)	12 days	YES *Vaginal Cytology*	Vehicle (saline) or 0.5% carboxymethylcellulos e	*no data*	Acetylcholine Δ; tryptophan glutamate Δ
Shetty and Gaitonde, 1980	Intact	Albino	EE = 1ug/kg NG = 10ug/kg	Oral gavage	Daily for 3 months	NO	Vehicle (0.1ml olive oil)	*no data*	Whole brain tis Δ; GAD ×; GAMA Pyridoxal Kina
Baker et al., 1977 θ	Intact	Unreported mouse strain	EE = 100µg/kg NETA = 200µg/kg	Injection (type unspecified)	Every 7 days for 42 days.	YES *Vaginal Cytology*	Vehicle (diestrua phase females)	Locomotor activity Δ	Free and total brain: tryptoph
Jori and Dolfini, 1976	Intact	Unreported rat strain	MES = 0.3mg/kg or 0.2mg/kg Lynestrol =5mg/kg NET=4mg/kg	Unreported	Daily for 4 days or daily for 30 days or; single injection	YES *Vaginal Cytology*	Vehicle (corn oil)	*no data*	Striatal DA lev
Ladinsky et al., 1976 θ	Intact	CD rats Albino-Swiss mice	MES = 0.06mg/kg, 0.2mg/kg 0.15mg/kg Lynestrol = 2.5 or 5mg/kg NET = 4mg/kg NER = 4mg/kg	Oral administration (method unspecified)	Daily for 30 days	YES *Vaginal Cytology*	Vehicle (corn oil)	*no data*	Acetylcholine choline acetylt
Srivastava and Laumas, 1975	Intact/ OVX	Wistar	NETA = 1mg / 2mg/ 3mg/ 5mg (oral); 8mg / 15mg / 25mg / 50mg (implant)	Oral gavage or subcutaneous implant (silastic capsule)	15 days and 42 days	YES *Vaginal Cytology*	Vehicle (oral olive oil; sham/empty implant)	*no data*	Ovarian weigh compensatory hypertrophy Δ
Marchi and Cugurra, 1974	Intact	Unreported rat strain	EE = 0.01mg/kg/0.1mg/kg NG = 0.1mg/kg/1mg/kg	Oral administration (method unspecified)	Daily for 1 or 2 months	NO	Vehicle (peanut oil)	*no data*	Hepatic and br monoamine ox Δ
Fregly, 1973 θ	Intact	Long-Evans (Included male rats)	MES + NER = 7.5 mg/kg (as Enovid®) or; EE = 1mg/kg / 2mg/kg NET = 7.5mg/kg / 15mg/kg	Voluntary Consumption (Purina laboratory chow containing Enovid®)	10 days; 25 days	NO	Vehicle (drug free chow)	Spontaneous running Δ; food intake Δ; NaCl preference Δ; fluid intake ×	Body weight Δ

Fregly, 1972	OVX	Holtzman	MES + NER = 7.5 mg/kg (as Enovid®)	Voluntary Consumption (Rockland Rat Diet containing Enovid®)	~8 months	NO	Vehicle (drug free chow)	Spontaneous running Δ	Heart, kidney, uterine weight blood pressure
Fregly et al., 1970	Intact	Holtzman	MES + NER = 7.5 mg/kg (as Enovid®) or; MES = 0.125mg/kg/ 0.54mg/kg Norethynodrel = 7.5mg/kg	Voluntary Consumption (Rockland Rat Diet containing Enovid®)	11-29 days	YES *Vaginal cytology*	Vehicle (drug free chow)	Spontaneous running Δ; food intake Δ	Body weight Δ

Note. Studies marked with θ indicate studies that did not use female rats but are still relevant to HC. Behavioral tasks and biomarkers marked with Δ indicate that there was a statistically significant difference between treatment groups receiving HC and the control group on this measure. Those marked with × indicate that no effect of HC was observed. **Abbrev.** (alphabetical): *Allo*: allopregnanolone; *BDNF: brain-derived neurotrophic factor; CPP: conditioned place preference; DA: dopamine; DHT: dihydrotestosterone; DSPM: dual solution plus maze task; E1: estrone; E2: 17β-estradiol; EE: ethinyl-estradiol; ENG: etonogestrel; EPM: elevated plus maze; FST: forced swim task; GABA: γ-aminobutyric acid; GAD: glutamic acid decarboxylase; GAMA-T: γ-aminobutyric acid transaminase; GDX: gonadectomized; LH: luteinizing hormone; LNG: levonorgestrel; mito: mitochondrial; MPA: medroxyprogesterone; MWM: morris water maze; NA: noradrenaline; NG: norgestrel; NER: norethynodrel; NET: norethindrone; NETA: norethindrone acetate; NOR: novel object recognition; NOP: novel place recognition; OFT: open field task; OVX: ovariectomy; P: progesterone; PMT: paced-mating task; RIT: resident-intruder task; SPBM: shock probe defensive burying; SPT: sucrose preference task; T: testosterone; T3: triiodothyronine; T4: thyroxine; TCA: the citric acid cycle; TSH: thyroid stimulating hormone; WRAM: water radial arm maze.*

Dosing.

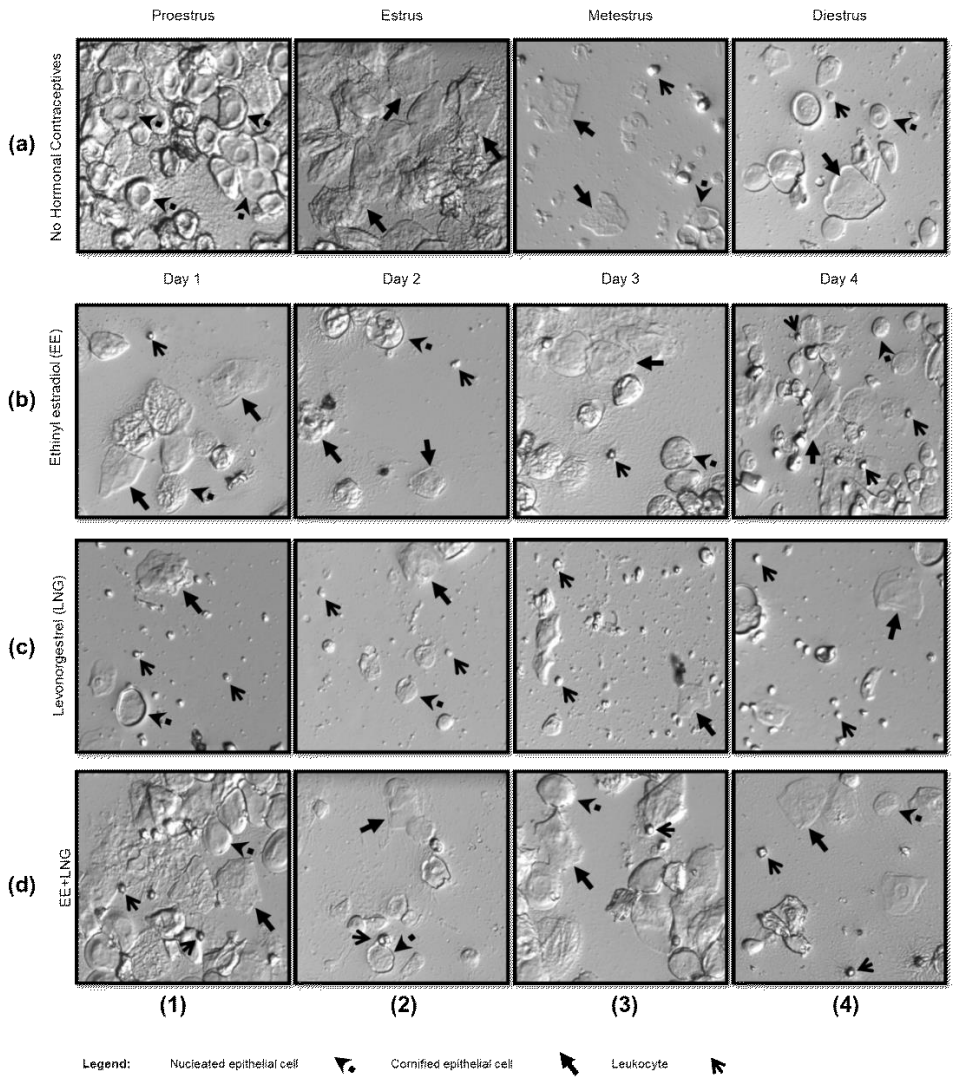
Selecting a dose. While there are detailed guidelines on how to translate drug doses between humans and rats (Nair and Jacob, 2016), these recommendations may fall short when studying HC. Free steroid hormones (endogenous or synthetic) have similarly low molecular weights, <400g/mol, and are non-polar and hydrophobic organic compounds (Payus et al., 2017). They readily permeate across phospholipid bilayers of cell membranes and the blood-brain barrier (Banks, 2012; Oren et al., 2004). EE is known to be active in the central nervous system (Fishman and Norton, 1977). These hormones do not act locally and are not always subjected to hepatic first-pass metabolism. Thus, traditional dosing strategies, such as using body surface area calculations (Nair and Jacob, 2016), do not apply to studies using steroid hormones (Fotherby, 1974). Seeing that we cannot use standard practices for dosing between rats and humans, alternate methods were developed.

One approach is to model the cessation of ovulation and cyclicity in female rats. Daily doses of 10-30μg/kg of EE can inhibit ovulation in female rats (Coelingh Bennink et al., 2008). Doses of 10μg/kg EE (Andrews et al., 2002) and 10μg to 30μg/rat/day of LNG are the lowest doses reported to achieve acyclicity in female rats (Kumar, 2000; Muhn et al., 1995;

Simone et al., 2015). We have shown that female rats administered 10μg/kg of EE, and/or 20μg/kg of LNG daily exhibit vaginal cytology indicating a persistent state of diestrus/metestrus (Fig. 1). This finding has been similarly reported by several others, albeit, using higher doses of EE and LNG (Graham and Milad, 2013; Hilz et al., 2021; Simone et al., 2015). Thus, doses approximating 10μg of EE and 20μg of LNG could be useful if the aim is to achieve a state of acyclicity. Others have reported the impact of other progestins on ovulation and vaginal cytology such as NET and NETA (Jones and Edgren, 1973; Muhn et al., 1995), NES (Kumar, 2000), CPA and DRSP (Muhn et al., 1995).

It is important to note, however, that the doses 10μg/rat/day of EE and 20μg/rat/day LNG may not affect other measures of cyclicity. For example, Simone et al. (2015) reported that these doses did not significantly alter circulating E2 relative to female rats in diestrus (Table 4). E2 levels were similarly not significantly impacted by administration of LNG (Graham and Milad, 2013; Hilz et al., 2021). However, these three studies measured serum E2 using enzyme-linked immunosorbent assay (ELISA) which may not be capable of detecting true differences in E2 levels at low circulating concentrations (<10pg/mL; Rosner et al., 2013; Schultheiss et al., 2018). Nevertheless, although there are observable alterations in vaginal cytology, those same doses may not necessarily reflect significant changes in circulating ovarian hormones (Table 4).

Figure 1.



Note. Images (20x magnification) of vaginal cytology taken using light field microscopy (Leica DM 5000) with differential interference contrast. Vaginal lavages (30µl of ddH20) were performed and estrous phases were staged according to Ajayi and Akhigbe, (2020) and McLean et al. (2012). Visual identification of cycle phase was done at 10x magnification. The first panel of images (a) displays samples from vaginal lavages performed on naturally cycling female rats that were not treated with hormonal contraceptives (HC). In female rats, the natural estrous cycle lasts between 4 to 5 days and can be broadly split in to three stages: proestrus, estrus, and diestrus (often subdivided into two stages: metestrus and diestrus). Panel (a1) displays vaginal cytology from the proestrus phase which typically lasts around 12 hours and can be identified by clusters of predominantly round nucleated epithelial cells. Panel (a2) displays vaginal cytology from the estrus phase which lasts between 24-48 hours and can be identified by clusters of cornified

enucleated epithelial cells. Panels (a3 and a4) display images taken during metestrus and diestrus, respectively. During diestrus, leukocytes can be present either in even distribution with nucleated and cornified epithelial cells, or as the predominant cell type. The diestrus stage can also be marked by a lower density of cells, as they tend not to cluster together, as well as the potential presence of mucus. Panels b, c, and d display images of samples from vaginal lavages performed on rats receiving (b) 10μg/kg ethinyl-estradiol (EE) alone; (c) 20μg/kg levonorgestrel (LNG) alone; (d) ethinyl-estradiol and levonorgestrel combined (same doses). Drugs were administered via subcutaneous injections between 8AM and 9AM daily for 21 days. As is shown in all images from panels b, c, and d, rats administered EE and/or LNG never show vaginal cytology that would indicate a state of proestrus (panel a1) nor estrus (panel a2). In all cases where EE and/or LNG are administered rats remain in a persistent state of metestrus/diestrus as indicated by the relatively even ratio of nucleated and cornified epithelial cells, as well as leukocytes (panels b, c, and d). Rats that received LNG alone (panel c) had an abundance of leukocytes that was not observed in any other treatment condition. It should also be noted that rats that received either EE alone (panel b) or EE+LNG (panel d) had a greater abundance of nucleated epithelial cells compared to rats administered LNG alone (panel c). The presence of vaginal mucus was observed in all rats exposed to HC as well as diestrus phase females.

Another approach to dosing HC in female rats requires the collection of serum and a means of accurately measuring serum hormone levels. Some have selected the dose of EE by working back from serum EE levels observed in humans after pill ingestion (Mennenga et al., 2015). Administering varying doses of EE and subsequently testing serum levels until they reach levels comparable to what is observed in humans. Mennenga et al. (2015) administered EE dosed at 0.125μg/day, 0.18μg/day, and 0.3μg/day to OVX female rats. Their 0.18μg/day dose was based on a 45-50μg/day regimen in an average woman weighing ~60-70kg (Curtis et al., 2014) adjusted for the weight of a rat (~0.25kg). They analyzed serum EE levels and found that 0.3μg/day achieved ranges of EE (23.17pg/mL ± 12.5pg/mL). These levels are comparable to those reported in the serum of women using oral contraceptives (OC) containing 35μg of EE early in the active pill phase (Devineni et al., 2007).

Mennega et al., (2015) report that with all three doses of EE used, the vaginal cytology reflected a state of steady estrus/metestrus. Rats also had serum levels of E1 and E2 below the detectable limits of 10pg/mL. However, the rats in this experiment were OVX, and therefore changes in vaginal cytology and serum hormones were likely due to the ovariectomy surgery rather than the hormonal treatment itself (Montes and Luque, 1988; Wise and Ratner, 1980). Nevertheless, working back from serum levels observed in humans is a promising method of modeling dosing of HC for future studies with gonadally-intact female rats. Tables 1 and 2 show

the peak serum concentration (C_{max}) and the time at which it was reached (T_{max}) for a variety of estrogens and progestins used in HC. These values can be used as targets for serum concentrations of estrogens and progestins that have not yet been studied in female rats. It should be kept in mind that basal levels of serum hormones could vary in relation to time of day and the assay used to quantify them.

Table 4) ***Serum LH, E2, P, and ALLO levels under different hormonal milieus.***

	LH *ng/mL*	**E2** *pg/mL*	**P** *ng/mL*	**Allo** *ng/mL*
Proestrus	1347±519 (I)	16.09±2.45 (VI) 79.5±8.5 (IV)	7.9±1.9 (IV) 23.17±5.09 (VI)	11±1.5 (XIII)
Estrus	5±1 to 8±2 (I)	2.657±1.02 (VI)	5.8±0.4 (IV) 6.8±0.8 (XV) 27.61±5.32 (VI)	13.3±1.2 (XV)
Diestrus/Metestrus	4.09±2.13 (III) 13±5 (I)	5.35±1.86 (VI)	7.8±0.4 (IV) 27.27±3.14(VI)	14.22±0.91(XI) 14±1.5 (XIII)
Ovariectomy	267.5-293.3 (II) >2000 (IV)	<LOD @ 10 (IV)	<5 (IV)	~0.5±0.1 (VII)
Males	3.2±0.7 (V)	<5 (V)	2.2±0.7 (V)	0.23±0.05ng/mL to 0.42±0.06 (VIII)
EE 10µg	<LOD @ 0.78 (III)	*d*= 0.38 (X) *	*NA*	*NA*
EE 30µg	*NA*	*d*= -1.15 (X) *	2.52±0.26 (XI)	8.53±0.65 (XI)
LNG 20µg	<LOD @ 0.78 (III)	*d*= 0.63 (X) *	*NA*	*NA*
LNG 50-60µg	*0.2*±0.1 (IX)	*d*= -0.67 (X) *	*NA*	*NA*
LNG 100-125µg	*NA*	*NA*	3.49±0.42 (XI)	8.39±0.46 (XI)
EE+LNG 10ug + 20µg	<LOD @ 0.78 (III)	*d*= 0.10 (X) *	*NA*	*NA*
EE+LNG 30ug + 60µg	*NA*	*d*= -1.31 (X) *	*NA*	*NA*
EE+LNG 30ug + 125µg	*NA*	*NA*	4.06±0.41 (XI) & 3.41±0.40 (XII)	8.10±0.78 (XI) & 11.15±0.54 (XII)

Note. Abbreviation: *L.O.D*: Limit of detection; Hormone levels are reported for gonadally intact male rats. Female rats given EE and/or LNG were also gonadally intact. EE and LNG were administered via subcutaneous injection. *E2 levels for citation (X) (Simone et al., 2015) are reported as the standardized difference (*Cohen's D*) between the EE and/or LNG treated group means and the mean of female metestrus-diestrus control rats (E2 = <10µg) as they were not reported as serum concentration. References (x): (I) Butcher et al., 1974; (II) Shaar et al., 1975; (III) Lacasse et al., 2022; (IV) Wise and Ratner, 1980; (V) Haim et al., 2003; (VI) Proaño et al., 2018; (VII) Genazzani et al., 2000; (VIII) Bernardi et al., 1998; (IX) Kumar, 2000; (X) Simone et al., 2015; (XI) Porcu et al., 2012; (XII) Follesa et al., 2002.

Dosing in combination. Some studies have examined the effects of EE administered alone (Andrews et al., 2002; Estrada-Camarena et al., 2004, 2003; Maher et al., 2021; Mennenga et al., 2015; Picazo et al., 2010; Vega-Rivera et al., 2013). Others have studied the

effect of progestins administered alone (Allaway et al., 2021; Graham and Milad, 2013; Hilz et al., 2021; Okojie and Oyekunle, 2014; Olanrewaju et al., 2013; Srivastava and Laumas, 1975). However, the majority of studies on HC in female rats examine the effect of EE and a progestin in combination (Table 3). It is rare that EE would be administered without a progestin given that EE unopposed could increase risks of negative outcomes such as venous thrombosis and other unwanted side effects (Amoozegar et al., 2015; Manzoli et al., 2012; Stanczyk et al., 2013). On the other hand, there are several formulations of HC that include exclusively the progestin component such as progestin-only pill formulations (POP), hormonal IUDs, hormonal patches or injections, vaginal rings, and emergency hormonal contraception (Dickey, 2021). Thus, if designing a model of HC with high ecological validity, whether EE is administered alone or in combination with a progestin can have important ramifications.

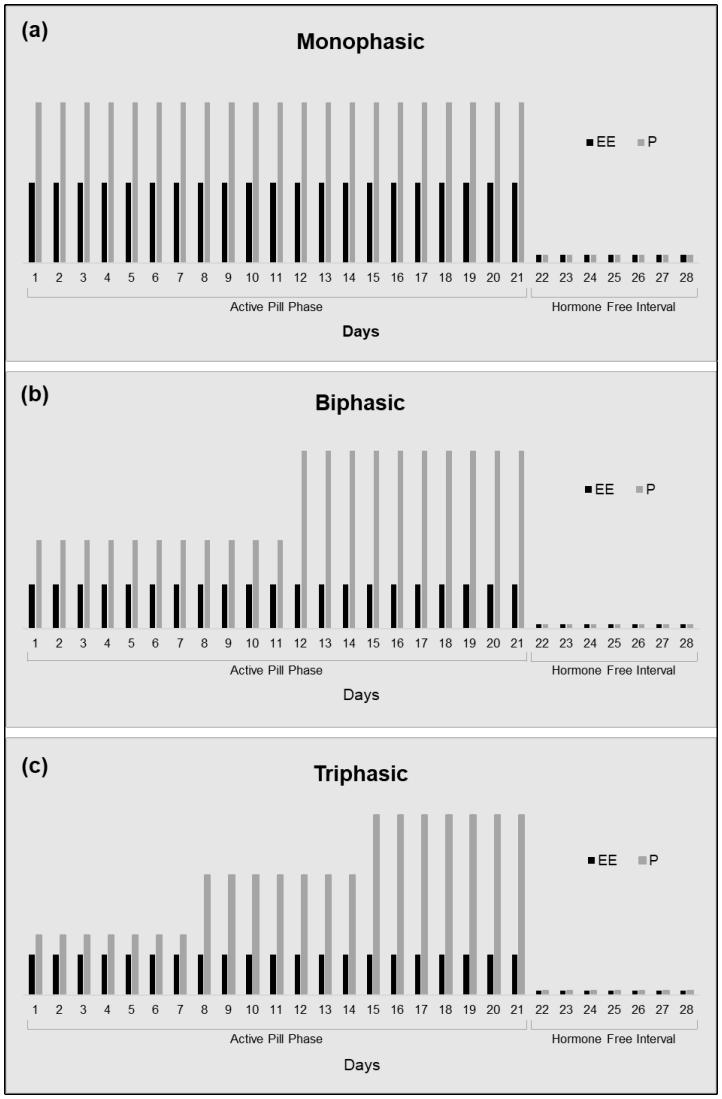
In our own behavioral experiments, rats given EE and LNG alone show behavioral effects opposite to rats administered the two in combination, at least within a spatial navigation paradigm (Lacasse et al., 2022b). Similarly, LNG has different effects on spatial memory when it is paired with E2 compared to when it is given alone (Koebele et al., 2021; Prakapenka et al., 2018). Thus, studies of these hormones in isolation may lead to conclusions about their effects that could change if the hormones were administered together.

Ethinyl estradiol / progestin ratio. A standard has not been established in terms of which dose could be a reasonable starting point for assessing other progestins that have not yet been tested in rodent models. Methods outlined in section 2.1. can be applied for selecting doses of progestin not previously tested in female rats That is, selecting doses which result in acyclic vaginal cytology (Jones and Edgren, 1973) or gonadotropin/ovarian hormone suppression; or by working back from serum concentrations observed in humans. Alternatively, progestin dose can be selected in relation to the selected dose of EE, which has been studied extensively in rats at doses ranging from 0.2μg/kg to 100μg/kg (Table 3).

The EE/progestin ratio for HC ranges from 1:5 to 1:50, with the most frequently used forms ranging from 1:10 to 1:30 (Dickey, 2021). After selecting a dose of EE, the progestin dose can be modelled according to the EE/progestin ratio of the particular HC formulation of interest. To date there are no behavioral or neurobiological experiments in female rats that examine the effects of, for example, the progestin DSG. A commonly prescribed formulation of HC contains 30µg of EE and 150µg DSG (Benagiano, 1989) which has an EE/progestin ratio of 1:5. To model this formulation, a dose of 10µg EE would require a dose of 50µg of DSG. Until further data are available, this is one potential approach to selecting progestin dose.

Modelling "phasic" effects by escalating doses. HC pill formulations can also differ in terms of dose escalation (Fig. 2). The majority of HC used are monophasic, meaning they contain the same dose of EE and progestin across all 21 active hormone pill days (Fig. 2a). Bi-phasic formulations escalate the dose once, midway through the active pill phase (Fig. 2b). Tri-phasic formulations escalate the dose twice, once every 7 days throughout the 21-day active pill phase (Fig. 2c). In the majority of multiphasic HC, only the progestin dose escalates, not the dose of EE (Dickey, 2021). For a comprehensive list of multiphasic HC formulations less often prescribed see (Dickey, 2021; Table 5).

Figure 2.



Note. The "phasic" component of HC across a 28-day oral contraceptive (OC) pill cycle for three different types of OC. The *phasicness* of an OC refers whether the hormones within the formulations are delivered at the same dose daily, or in fluctuating doses throughout the 21-day active pill phase. In the bar graphs above, dark bars indicate the dose of the ethinyl estradiol (EE) component of OC, and light bars indicate the progestin (P) dose. (a) Monophasic OC formulations deliver the same dose of EE and P daily. (b) Biphasic OC formulations deliver the same amount of EE across 21 days of the active pill phase, but the P dose increases, once, between the first and second half of the active pill phase. (c) In triphasic OC formulations EE dose remains the same throughout the 21-day active pill phase, while the progestin dose increases every seven days of the active pill phase. Note that in all forms (monophasic, biphasic, and triphasic), EE and P are presented for 21 days, and followed by a 7-day hormone free interval. In some cases, active pill phases may be 24 days long, and hormone-free intervals may be up to 10 days long (Dickey, 2021).

If the aim is to study the "phasic" effects of HC, it is possible to use a strategy similar to that outlined in the previous section (section 3.2.1). The dose calculation required to model phasic pill formulations is less straight forward than simply modelling a monophasic

EE/progestin ratio. One important factor to consider is the degree to which the progestin dose increases, *each time it increases*. For example, a popular triphasic formulation contains EE at 0.025mg, and the progestin, NOR, at three different doses (0.180mg, 0.215mg, and 0.250mg; Hampton et al. 2001).To model this formulation, the multiple between dose 1 (0.180mg) and dose 2 (0.215mg) is a factor of 1.194x. Yet, the multiple between dose 2 (0.215mg) and dose 3 (0.250mg) is slightly less at 1.163x. It should also be noted for modelling phasic HC in rats, that the phasic increases in progestin dose should be fitted to the 4-5-day estrous cycle, rather than a 28-day human menstrual cycle. To continue with our example above, EE+NOR could be administered *triphasically* by increasing progestin doses daily for three consecutive days, followed by hormone withdrawal for 1-2 day(s), repeated over multiple cycles. To our knowledge, no rodent experiments to date have modeled the phasic component of HC.

Dose-dependent effects of contraceptive hormones.

It is well established that the behavioral and neurobiological effects of endogenous E2 in female rats are dose-dependent (Engler-Chiurazzi et al., 2011; Lacasse et al., 2022b; Pisani et al., 2012, 2016; Quinlan et al., 2008, 2013; Sinopoli et al., 2006; Talboom et al., 2008; Uban et al., 2012; Zeibich et al., 2021; Zurkovsky et al., 2011). T has also been reported to impact spatial learning in a dose-dependent manner in male rats (Spritzer et al., 2013; Zhang et al., 2020). When designing studies on the behavioral neurobiology of HC, it is therefore important to consider that effects are likely dose dependent.

EE and LNG have also shown dose-dependent effects on the brain and behavior. Estrada-Camarena et al. (2003) found that acute injections of EE (2.5µg or 5µg) reduced immobility on a forced-swim task, whereas a dose of 10µg did not. Simone et al. (2015) found decreased anxiety-like behaviors in an elevated plus-maze (EPM) when administering EE at 10µg and LNG at 20µg daily for 21 days. Yet, at higher doses (EE 30µg/day / LNG 60µg/day) anxiety-like behaviors such as freezing increased. Others have reported that chronic doses of 30µg of EE and 125µg of LNG (Follesa et al., 2002; Porcu et al., 2012) or LNG alone (125µg;

Porcu et al., 2012) increase anxiety-like behaviors in an EPM. Thus, the effects of EE and LNG on anxiety-like behaviors are dose-dependent.

EE also has dose-dependent effects on tests of spatial working memory. For example, 0.3μg of EE impaired performance on a spatial working memory task, 0.18μg of EE transiently impaired it, and 0.125μg of EE did not impact performance (Mennenga et al., 2015). In humans, EE dose has an inverse correlation with mental rotation such that performance is impaired with increasing doses of EE (Beltz et al., 2015). Thus, in rats and in humans, increasing the dose of EE may impair performance on tasks related to spatial cognition.

In summary, HC effects on behavior can be dose-dependent but are also task-dependent. Anxiety/depression-like behaviors are impacted differently by low and high doses of EE (Estrada-Camarena et al., 2003; Simone et al., 2015). Whereas behaviors related to spatial cognition are increasingly impaired as EE doses increase (Beltz et al., 2015; Mennenga et al., 2015).

Routes of Administration

When it comes to route of administration (ROA) there are several options to consider, each of which has a cost and a benefit.

Enteral ROA. Given that many formulations of HC are delivered orally, delivering the drug orally to a rat is important in terms of the model's ecological validity. When administered orally, the synthetic hormones used in the experiment will be subjected to, for example, the highly acidic pH of the stomach and hepatic first-pass metabolism (see Stanczyk et al., 2013 for a review of EE metabolism in humans). In women using oral formulations, the drug is metabolized by hepatic CYP enzymes such as 3A4 (Stanczyk et al., 2013). However, rats metabolize drugs and steroids differently than do humans (Akpoviroro et al., 1981; Fotherby, 1974; Jenkins and Fotherby, 1981; Nair and Jacob, 2016). At the surface, an enteric ROA seems ecologically valid, yet, it may result in different serum levels of the drug in rats compared to humans.

One approach to using oral administration in rats could be to dissolve HC into a palatable (sucrose) solution or gel and allow the rats to consume it (Fregly, 1972; Fregly et al., 1970). This may be the most ecologically valid ROA given that the subject is voluntarily consuming the drug orally. However, the rats willingness to consume the mixture is an important variable. Ovarian hormones influence appetite (Asarian and Geary, 2006), and therefore may affect the voluntary consumption of sucrose solutions. An alternative is to administer the HC using oral gavage. Oral gavage administers the drug directly into the stomach of the rat (Turner et al., 2012). Although this technique does achieve the goal of accurately dosing HC orally in rats, it does so at the cost of being stressful (Bonnichsen et al., 2005; Brown et al., 2000; Walker et al., 2012). Unless studying the acute effects of synthetic contraceptive hormones, rats would then have to receive gavage daily. Thus, while oral gavage may be a useful technique, it may be too invasive to be used in chronic experiments on HC, especially those where the behavior may be influenced by stress or anxiety.

Parenteral ROA. The majority of studies on HC in female rats use subcutaneous (s.c.) injections as their primary ROA (Table 3). Although less ecologically valid, s.c. injections are less invasive and are more straight-forward compared to s.c. capsule implants which require surgery. Another ROA is the implantation of osmotic pumps or silastic capsules. These have been used to study the impact of E2 in female rats (Almey et al., 2013; Mannino et al., 2005; Talboom et al., 2008). Using implants is useful as they release low doses of a drug, chronically, over an extended period of time. This may translate to non-oral routes of administration for HC often used by women i.e., hormonal IUD, vaginal rings, and patches. However, this comes with the complication that implants require surgery. Moreover, if the exposure time of the experiment exceeds the volume that an implant is able to hold, the use of implants may require additional surgeries over time to add new implants. What is clear is that each ROA has a benefit and a cost as it relates to balancing experimental simplicity with ecological validity. While some routes benefit from being more translatable to humans (e.g., voluntary consumption, oral gavage, and

implants), they come with the cost that may they introduce confounds that could impact the animals' behavior such as stress and anxiety.

Timing.

Exposure time. The number of days the rat is exposed to HC, once again, depends on the aim of the experiment. Most oral HC provide 21 days of hormonally active pills (pills which contain the synthetic hormones), followed by 7 days of hormone-free pills (Dickey, 2021; Fig. 2). Thus, one approach has been to expose female rats to HC daily for 21 consecutive days (Follesa et al., 2002; Simone et al., 2015; Wusu et al., 2021). However, as we will see in the next section (5.2.), 21 days of chronic exposure reflects a longer period of development in a rat than 21 days would reflect in women.

Alternatively, the 21-day active / 7-day hormone-free component of HC can be modeled by mimicking the proportion of time spent using active or hormone-free pills. For example, there are 28 days for one pill cycle, thus, 21 days (75%) of the pill cycle contain active hormones and 7 days (25%) contain no active hormones. Thus, one could use these same proportions fitted to the 4-day rat estrous cycle. That is, 3 days of HC (75%), followed by a single day of hormone-free treatment (25%). This can be repeated for 16-21 days which allows for roughly four to five cycles to elapse as done by Raval et al. (2012).

The duration of exposure to HC will likely have some impact on experimental outcomes (Braden et al., 2011; Nwakanma et al., 2021). Researchers should exercise caution when designing experiments with long exposures to HC. Extended exposures could lead to significant (detrimental) physiological changes in rats treated with HC compared to those in the control conditions. For example, in humans, extended exposure to low-dose combined oral contraceptives can have several negative ramifications on, for example, endothelial structure and function (Heidarzadeh et al., 2014; Williams and MacDonald, 2021), and risk for venous thromboembolism (Amoozegar et al., 2015). In rats, extended exposure to EE has also been shown to increase endothelial permeability in the aorta and in cerebral capillaries (Gammal and

Zuk, 1980), and can significantly alter brain energy metabolism (Diaz and Raval, 2020). With extended exposure to HC, the number of subtle physiological changes like these will compound, and the convergence of all of these physiological changes will make interpretating the effects of HC more difficult over time unless explicitly controlled for.

Exposure time will differ depending on which formulation of HC is being modeled. For example, in continuous pill formulations, hormonally active pills are consumed daily for ~3 months (84 days), followed by a 7-10 day hormone-free interval (Dickey, 2021; Krishnan and Kiley, 2010). When modelling longer acting formulations or exposure beyond a single pill cycle, it will be necessary to expose rats to HC for longer periods of time. In such cases, exposure time should also be considered in light of ROA. Daily exposure over long periods (> 30 days) may be best suited to a ROA such as s.c. implants or voluntary consumption. To date, no experiments have examined the chronic effects of HC in female rats for longer than 120 days.

When examining extended exposure to HC (> 1 day), an acute dose control should be considered. This control group is otherwise treated identically to those receiving the HC chronically, however, are only administered the active hormones a single time on test day. While it is important to test for the acute effects of HC, this is primarily to control for acute vs. chronic effects of each hormone component. This cannot be done in experiments using, for example, certain learning and memory tasks that occur across several days (e.g., Morris Water Maze (MWM), Water Radial Arm maze (WRAM), and Barnes maze). Nevertheless, with translation to humans in mind, it is rare that women would be exposed to these synthetic hormones acutely, other than in cases where they are used in an emergency contraception formulations.

Exposure timing. It is important to consider exposure time in light of exposure *timing*, or the developmental window in which the synthetic hormones are presented. Rats can live for up to ~3 years (Agoston, 2017), and reach sexual maturity ~84x faster than humans. Thus, the rate of development in rats is much quicker than humans (Agoston, 2017; Quinn, 2005). Exposure

time must be selected with this temporal difference in rate of development in mind. It is therefore important to consider A) the developmental window in which HC are first presented, and B) that the same amount of exposure time for humans reflects a much longer developmental window in rats.

Many young women begin taking HC during or shortly after puberty (Dickey, 2021). Between puberty and adulthood, the brain undergoes organizational changes that are intrinsically tied to steroid hormones (Diviccaro et al., 2021; Juraska and Willing, 2017; McCormick and Mathews, 2010, 2007; Rehbein et al., 2020). Thus, an important consideration is whether HC are impacting the developing adolescent brain and whether there are differences between those who begin using HC in adolescence as opposed to adulthood (Cahill, 2018). In order to properly model the adolescent period in female rats, it is important to consider some of the ways sexual maturity differs between rats and humans. For example, it takes ~50 days for a female rat and ~11.5 years for a woman to reach sexual maturity (Agoston, 2017; Otto et al., 2015; Quinn, 2005; Sengupta, 2013). Rats could therefore be exposed to HC during adolescence or only after reaching adulthood.

Examining the impact of HC during specific developmental windows in rats will allow for future studies to uncover how HC may impact adolescent neurodevelopment. Clancy et al. (2001) provide a detailed guide for translating developmental time between rats and humans, specifically, as it relates to the development of particular brain regions. This resource is useful for researchers interested in studying the impact of HC on the maturation of specific brain regions. To date, no studies have examined the neurodevelopmental effects of exposure to HC in female rats. There are several elements of neurodevelopment that are intricately linked to steroid hormones during adolescence. Thus, it is important that we strengthen our understanding of the potential impact of exposure to potent synthetic hormones during adolescence. This can be made possible by considering exposure timing when studying the effect of HC in female rats.

Validating models of contraception.

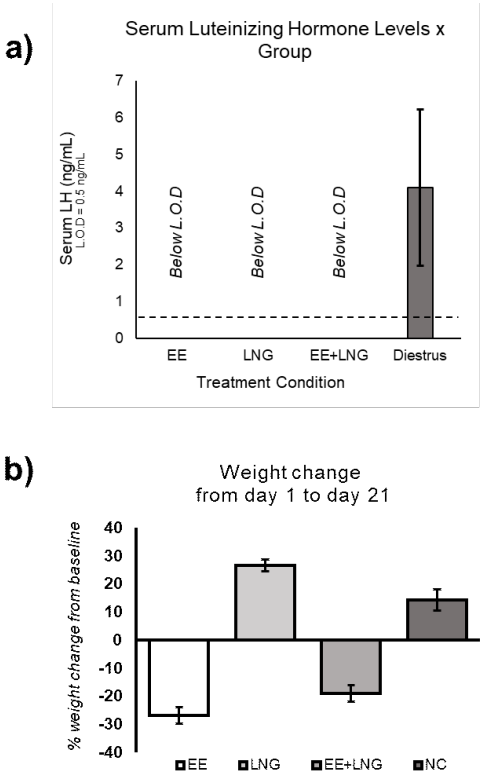
After selecting the dose, ROA, and exposure time for the experiment, it is important to verify whether these specifications succeed in inhibiting ovulation or reaching a state of acyclicity. Vaginal cytology and HPG suppression are two converging lines of evidence that the model of HC is effective at producing this result.

Vaginal Cytology. Vaginal cytology can be used to identify what stage of the estrous cycle a rat is in and can confirm that the dose / ROA / exposure time selected is having a contraceptive effect i.e., inhibition of ovulation / acyclicity. In female rats, the estrous cycle typically lasts between 4 to 5 days. The cycle can be broadly split into three stages: proestrus, estrus, and diestrus (diestrus can be further subdivided into two stages: metestrus and diestrus; Fig 1). The proestrus stage typically lasts around 12 hours, the estrus stage lasts between 24-48h, whereas the diestrus stage can last anywhere from 48-72h. The diestrus stage can also be marked by a lower density of cells, as they tend not to cluster together, as well as the potential presence of mucus. See Fig. 1 for further description of these phases. Female rats receiving 10µg/kg EE, 20µg/kg LNG, or both combined show vaginal cytology that reflects a persistent state of diestrus (Figure 1b, c, d). The persistent state of diestrus/metestrus in gonadally intact female rats receiving EE and/or LNG has been reported by others (Graham and Milad, 2013; Hilz et al., 2021; Simone et al., 2015). We also observed that LNG produces a predominance of leukocytes (Fig 1c).

HPG axis suppression. Another assay for whether or not the selected dose / ROA / exposure time is inhibiting ovulation is to measure suppression of the HPG axis. A model of HC should successfully inhibit GnRH, and result in very low levels of gonadotrophic and ovarian hormones. For example, serum LH levels are heavily suppressed following 10µg/kg EE, 20µg/kg LNG, or both combined (Fig 3a). Ovarian hormone levels can also be measured to detect HPG suppression. It was demonstrated that levels of E2 and P are lowered after administration of higher doses of HC in female rats (Table 4). As can be seen in Table 4, there

is still a lot of missing information on the effects of HC on circulating hormone levels in the female rat.

Figure 3.



Note. (a) Serum luteinizing hormone (LH) levels in rats receiving either 10µg/kg ethinyl-estradiol (EE) alone; 20µg/kg levonorgestrel (LNG) alone; same doses of EE and LNG combined; and cycling female rats in diestrus. Drugs were administered via subcutaneous injections daily for 21 days. LH levels in rats that received EE and/or LNG were below the limit of detection (L.O.D.) of the assay (0.5ng/mL). *Procedure:* Within 15 minutes of behavioral testing rats were euthanized and blood samples were collected. Samples were immediately centrifuged, and serum was isolated and stored at -20 ◦C. Serum LH concentration (ng/mL) were analyzed via a commercially available enzyme-linked immunosorbent assay (ELISA) from Abnova (Catalog number: KA2332). This kit reports a sensitivity of 0.5ng/mL and an L.O.D. of 0.5ng/mL. Intra-assay reliability = 6.05%CV, inter-assay reliability = 7.68%CV. (b) Percent changes in body weight from baseline in gonadally-intact female rats receiving the same treatment listed in (a). Rats were 2-3 months old female Long-Evans rats and weighed ~240g at baseline. Rats in all treatment conditions each received ~30g/day of low-phytoestrogen rat chow (Teklad Global Rat Chow®). Rats administered EE alone or EE+LNG lost a significant percentage of body weight. Those administered LNG or that were cycling gained weight over 21 days.

Body weight. Bodyweight changes on their own should not be considered as a validation of contraception. Yet, along with other measures, it is a converging line of evidence

that the HC are exerting a physiological effect (Jones and Edgren, 1973). If no brain-related or behavioral outcomes are impacted, altered body weight may at least confirm that the hormones were exerting a physiological effect. For example, female rats fed the same amount of food daily (~30g) and exposed to EE and/or LNG have changes in body weight from day 1 to day 21. As shown in Fig. 3b, 10µg/kg of EE alone or 10µg/kg EE paired with 20µg/kg of LNG significantly reduced body weight by ~25-30%. Whereas rats given 20µg/kg of LNG gained weight over the 21 days, similar to what we observed in naturally cycling females (Fig 3b). This may not be a measure that confirms the contraceptive model, *per se*. However, ~25-30% weight loss is observed in rats exposed to EE relative to naturally cycling controls. Several other studies have observed changes in body weight in rat models of HC (Allaway et al., 2021; Andrews et al., 2002; Braden et al., 2011; Fregly, 1973; Fregly et al., 1970; Jones and Edgren, 1973; Koebele et al., 2022; Lacasse et al., 2022b; Maher et al., 2021). Therefore, weight loss may serve as a proxy for establishing that EE is exerting a physiological effect.

To be clear, we do not know exactly *how* EE may be impacting body weight. Endogenous E2 has several effects that could impact weight loss. For example, E2 is important in regulating energy metabolism (Barros and Gustafsson, 2011; Mauvais-Jarvis et al., 2013), energy expenditure (i.e., locomotion; Cushing et al., 1995; Fahrbach et al. (1985), food intake (Boswell et al., 2006; Rivera and Stincic, 2018), food palatability (Clarke and Ossenkopp, 1998), appetite for food (Asarian and Geary, 2006), food-reward (Richard et al., 2017) and even water intake (Santollo et al., 2021). Thus, there could be many factors at play when it comes to the weight loss effect we observed when exposing rats to EE. This is also something important to note when using food as a reward in behavioral paradigms when administering EE.

Control Group.

The decision of what control group to include will largely depend on which component of HC are being modeled. If controlling for some of the known hormonal effects of HC, then the following control groups may be useful to consider.

Controlling for low circulating pituitary and ovarian hormones.

Diestrus females. Several studies have used female rats in the metestrus/diestrus phase as their control group in studies of HC (Baker et al., 1977; Graham and Milad, 2013; Hilz et al., 2021; Simone et al., 2015). Female rats in diestrus serve as a natural control for the primary mechanism of action of HC (section 2.1.). Recall that HC work by inhibiting the production of hypothalamic GnRH, which subsequently results in a significant reduction in LH and FSH, and eventually E and P. Levels of these hormones are typically substantially lower in women using HC compared to naturally cycling women (El Etreby et al., 1979; Hampson, 2020; Scott et al., 1978). During diestrus, pituitary gonadotropins and ovarian hormones reach a natural low (Staley and Scharfman, 2005) which offers the opportunity to compare rats with suppressed levels to those with naturally lower levels. However, a diestrus phase control does have its pitfalls. Although rats exposed to HC might have suppressed levels of E2 and P, they also have potent synthetic hormones in circulation whereas diestrus phase controls do not. Thus, outcomes in rats exposed to HC could be due to the synthetic hormones, rather than the ovarian hormone suppression necessarily.

Ovariectomy. There is little homogeneity when it comes to circulating hormone levels in female rats, even within the same phase of the estrous cycle circulating hormone levels will vary. Thus, to control for this hormonal variability it may be wise to use OVX female rats as a control group. While this allows for more control over the variability of ovarian hormones from rat to rat, using an OVX control group also has its pitfalls. Its application has limited translational value for studies of HC in female rats. Using bilateral ovariectomy is more translationally relevant for models of surgical menopause (Acosta et al., 2009; Koebele and Bimonte-Nelson, 2016; Long et al., 2018; Zeibich et al., 2021). For a detailed review of rodent models of menopause see Koebele and Bimonte-Nelson (2016).

In some cases, it may be useful to ovariectomize all rats to directly compare hormone replacement treatments (Estrada-Camarena et al., 2004, 2003; Lemus et al., 1992; Maher et al.,

2021; Mennenga et al., 2015). For example, directly comparing the effects of synthetic EE to those of endogenous E2 (Estrada-Camarena et al., 2004, 2003; Maher et al., 2021; Mennenga et al., 2015), comparing a progestin to endogenous P (Braden et al., 2010), or comparing multiple progestins to each other (Braden et al., 2017). These strategies could be useful in terms of gaining an understanding of quantifiable differences in effects exerted by endogenous and synthetic hormones relative to one another. However, previous studies in female rats have shown that rats receiving ovariectomy differ significantly from naturally cycling female rats on several neurochemical, endocrine, and metabolic endpoints, even when replaced with E2 (Kirshner et al., 2020; Long et al., 2019, 2018). For this reason, studies on HC that use OVX female rats have limited translational value for humans.

The primary reason why ovariectomy has limited application in models of HC is that it prompts a significant rise in circulating LH in young rats (Wise & Ratner, 1998), which continues to increase for at least 24 days after the surgery (Shaar et al., 1975). However, administering HC actually causes circulating LH levels to decline significantly (Fig 3a). Thus, at the level of gonadotropins, ovariectomy and HC have very different impacts. Using an OVX control may therefore inadvertently impact tests that are sensitive to LH levels (Bhatta et al., 2018; Blair et al., 2015; Bohm-Levine et al., 2020) which could lead to inaccurate conclusions about the impact of HC. The use of an OVX control may not be a strong comparison given that rats receiving HC are still gonadally-intact despite having low levels of circulating pituitary and ovarian hormones. Thus, comparing rats exposed to HC to OVX controls may artificially inflate any observable differences between these groups.

Controlling for GnRH suppression. Another option is to control directly for the mechanism of action of HC by suppressing GnRH (see section 2.1.). This would entail using a GnRH receptor antagonist such as cetrorelix or ganirelix (Engel and Schally, 2007; Mező and Manea, 2009). Antagonizing GnRH receptors could help to dissociate whether any effects observed with HC are indirectly due to the suppression of the HPG axis, or whether they are

directly due to the impact of the synthetic hormones themselves. To date, there have been no studies on HC in rats that have controlled for GnRH suppression in this way.

Controlling for androgenicity. As outlined in section 2.3.2., many of the progestins used in HC are androgenic and have a high binding affinity for AR, particularly those derived from 19-nor testosterone. As such, the inclusion of male rats as a comparison group could address the androgenic effects of certain progestins. For example, women using androgenic formulations of HC show improved performance in a mental rotation task (Wharton et al., 2008), and in latency on spatial navigation tasks (Gurvich et al., 2020). Both mental rotation and latency in spatial navigation show a significant sex difference such that males tend to outperform females (Brake and Lacasse, 2018; Hampson, 2018). Female rats administered an androgenic progestin may therefore perform more similarly to males on tasks that have established sex differences. Thus, inclusion of male controls may help elucidate if the effect of HC on task performance is related to the progestins androgenicity.

Following the same logic, to investigate the potential androgenic effects of a progestin, control females can be treated with androgens such as T or DHT to see if the effects mirror what is observed with the progestin. For example, Lemus et al. (1992) administered male rats with either E2 and DHT or E2 and LNG. The authors noted similar androgenic effects of both LNG and DHT in castrated male copulatory behavior. Comparing treatment with androgenic progestins directly to the effect of androgens may also be an effective control. This method could also be applied to, for example, the administration of E1 or E2 to examine estrogenic activity, or P and its metabolites for progestational activity.

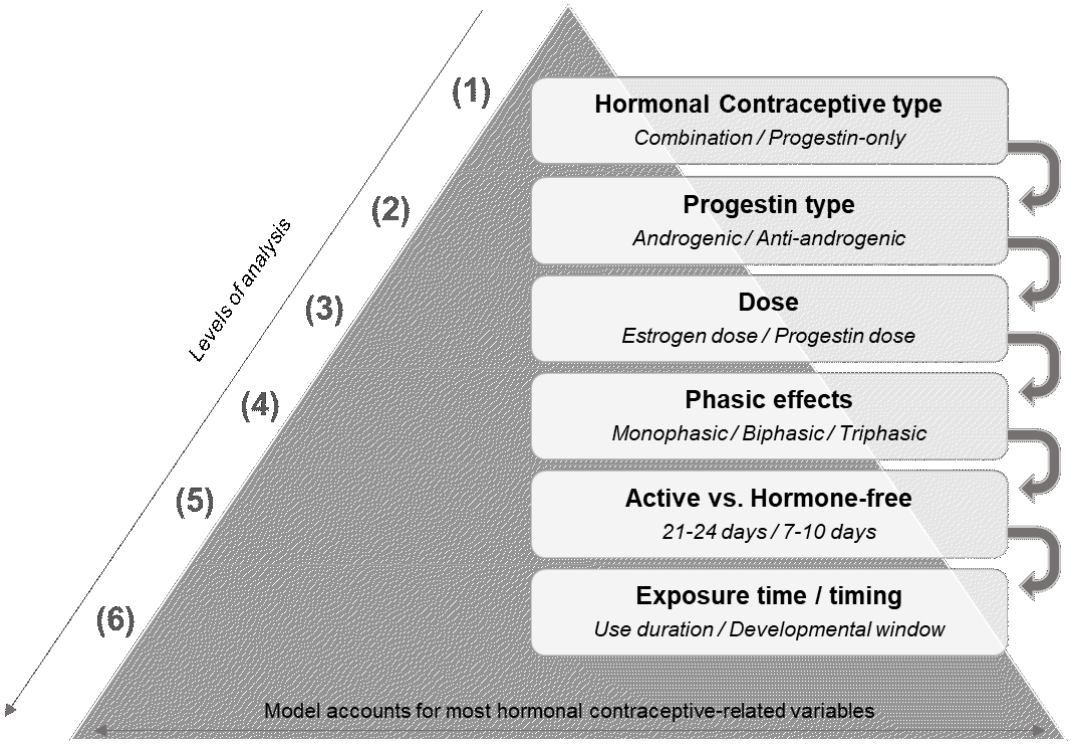
Framework for modeling HC in female rats.

Above we have outlined the essential components for designing a model of HC in female rats. As we have shown, there are several important decisions that must be made regarding dosing, ROA, exposure time/timing, and control groups. However, these elements are the most basic parts of designing a model of HC in female rats. Here, we reference findings in humans

that inform us on what components of HC may be appropriate to examine when using rat models.

Levels of analysis for studies on HC. Six levels of analysis were determined from studies in humans as relevant factors to consider when modeling HC in rats. Figure 4 illustrates a framework that describes each of these levels of analysis. A single study may be able to incorporate more than one level, but to capture all six levels of analysis within a single experiment would be a great challenge. Each level encompasses those that preceded it. For example, to arrive at the 3rd level of analysis (dose), assumes that levels (1) type of HC and (2) progestin type have already been considered. The flowchart provided in figure 4 works for current formulations of HC and can be applied to most outcome variables.

Figure 4.



Note. Six levels of analysis suggested when designing studies of hormonal contraceptives in female rats.

HC Type. The first useful comparison is to contrast the different types of HC. For example, oral versus non-oral forms, and those which contain estrogens and progestins versus progestin-only. For example, many women use OC, while others use IUD or other non-oral routes (i.e., vaginal ring, patch, injection; Dickey, 2021). Thus, a basic level of assessment is whether this simple difference in ROA can impact the brain and behavior differently. Next, there could be differences due to HC that are combined oral contraceptives (COC), i.e., formulations that contain both EE and a progestin, compared to progestin-only pills (POP). In human epidemiological studies, POP produce fewer mood-related side effects (Schaffir et al., 2016), and progestin-only IUD users carry a greater risk of developing depression (Skovlund et al., 2016).

Progestin type. As described in section 2.3.2. progestin molecules can have androgenic or anti-androgenic effects. In human studies of HC, the androgenicity of a progestin has been an important factor moderating effects on brain structure (Pletzer et al., 2016, 2019a), brain function (Menting-Henry et al., 2022; Pletzer et al., 2014a, 2016), mental rotation ability (Griksiene et al., 2018; Griksiene and Ruksenas, 2011; Wharton et al., 2008), verbal abilities (Beltz et al., 2015; Griksiene and Ruksenas, 2011), spatial navigation (Gurvich et al., 2020), facial emotion recognition (Gurvich et al., 2020; Menting-Henry et al., 2022), attention (Pletzer et al., 2014b), and stress-induced cortisol (Herrera et al., 2019).

Dose. As outlined in section 3, dose is an essential element to consider in models of HC. In humans, dose has been shown to inversely correlate with mental rotation performance, such that performance worsens as EE dose increases (Beltz et al., 2015).

Phasic effects. There are differences between monophasic, biphasic, triphasic, and continuous pill formulations. For example, women taking monophasic pills outperform naturally cycling women on a mental rotation task, but those taking triphasic pills do not

(Beltz et al., 2015). Moreover, women taking triphasic OC also showed a deficit in expressional fluency compared to naturally cycling women and those taking monophasic OC (Beltz et al., 2015). Otherwise, there has been no research on the phasic effects of HC on the brain.

Active pill and Hormone-free interval. Most oral forms of HC contain 21-24 active pills followed by 7-10 “sugar pills” or pills which do not contain any active hormones (Dickey, 2021). Thus, an important level of analysis is to compare groups based on whether they have received the active hormones or not. For example, in human studies, certain effects of HC on brain function and cognition are observed only when taking the active pill (Herrera et al., 2020; Mordecai et al., 2008; Nasseri et al., 2020; Peragine et al., 2020; Petersen et al., 2015).

Exposure Time / Timing. As noted in section 5, it is important to consider the amount of time exposed to HC and the developmental window during which the exposure occurs. Some studies in humans have shown an impact of increased exposure time, or duration of use, on brain structure (Pletzer et al., 2019a) and on cognition (Egan and Gleason, 2012; Marečková et al., 2014). Only three studies have compared the impact of HC on the brain from adolescence to adulthood (Marečková et al., 2014; Sharma et al., 2020a, 2020b). Indeed, there are structural and functional differences in the brains of those who began taking HC during adolescence that are not observed in those who began taking HC during adulthood (Marečková et al., 2014; Sharma et al., 2020a, 2020b). Exposure to HC during adolescence in women alters behavior and physiology related to stress reactivity (Sharma et al., 2020b), and facial recognition (Marečková et al., 2014). It should also be noted that an association between depressive/mood disorder symptoms and HC is primarily observed in young rather than older women using HC (de Wit et al., 2020; Mizutani et al., 2014; Skovlund et al., 2016). Overall, very little is understood about

the neurodevelopmental impact of HC in humans during adolescence (Cahill, 2018), but this is an important consideration for future studies.

Limitations of this framework.

The framework we have outlined does not account for variables that do not fit neatly into a broad category that applies to all HC. For example, it cannot account for those who discontinue HC for brief periods or who switch between types of HC across their lifespan (Pletzer et al., 2019a; Taylor et al., 2021). It does not account for nonadherence to usage guidelines (Leahy et al., 2015; Martínez-Astorquiza-Ortiz de Zarate et al., 2013; Molloy et al., 2012), or lapses in usage (Zapata et al., 2013). Our framework also cannot account for those who choose to skip the inactive pill phase entirely. For example, 73% of women will skip the inactive pill phase at least once, and 38% do so regularly (Picavet, 2014). Our framework, therefore, assumes perfect adherence to the recommended usage guidelines of HC.

This framework cannot speak to the enduring effects that HC may have on the brain and behavior. For example, Graham and Milad (2013) noted that with the removal of treatment with HC rats restored normal estrous cycling within 4-5 days. All studies of HC in rats, and the majority of studies in humans, assess subjects that are exposed to HC at the time of testing/sample collection. This begs the question of how long neurobiological or behavioral effects caused by HC might endure after administration is discontinued. In humans, some research suggests that taking HC earlier in life leads to increased grey matter volume in the basal ganglia (Pletzer et al., 2019a) and to improved cognition (Egan and Gleason, 2012) even many years after discontinuation.

There are also broader limitations that relate specifically to the use of rats to model human use of HC. The human menstrual cycle differs in many ways from the rat estrous cycle. For example, hormones like E2, P, LH, and FSH rise and fall in circulation at different points in the human and rat cycles (Staley and Scharfman, 2005). Similarly, behavioral responses associated with the cycle phase are sometimes different or opposite when comparing findings

from rats and women (Hussain et al., 2016 vs. Lacasse et al., 2022). Additionally, certain physiological effects of HC may not be well represented in a rat model. For example, in humans, many formulations of HC produce a significant rise in SHBG, which further suppresses levels of sex steroids in circulation (Kuhl, 2005; van der Vange et al., 1990; Wiegratz et al., 2003). Rats produce little hepatic SHBG (Laurent et al., 2016; Wang et al., 1990), and one study reported that, in rats, binding of LNG is confined to albumin only (Srivastava et al., 1984).

Facilitating translational HC research.

Researchers who study humans can facilitate translation via animal models in a few different ways. Future experiments in humans should be designed in light of the complexities of the diverse pharmacology of HC and their mechanism of action. Hampson (2020) outlines methodological considerations which account for the pharmacology and physiology of HC in human studies. Future studies should also aim to recruit samples with high homogeneity among types of HC rather than large samples that include inconsistent and diverse types. For example, some have recruited women taking one specific brand of HC (Bianchini et al., 2018), and others have recruited women taking HC that have similar physiological effects (i.e., anti-androgenic; Griksiene et al., 2018). Only by studying individual progestins / formulations will we make real progress in our understanding of the different mechanisms underlying the effects of HC.

Whenever possible, collaboration among researchers who work with humans and those who work with animals is encouraged. Some experiments have examined the impact of HC on similar behaviors and biomarkers simultaneously in both humans and in rats (Follesa et al., 2002; Graham and Milad, 2013). This type of translational design is ideal for interrogating mechanisms underlying the effects observed in humans. However, this design requires behavioral tests that are applicable to both rats and humans and may therefore not be well suited to all aspects of human behavior. For those interested in this translational design, spatial navigation and fear extinction are two behaviors that have validated assessments in both

rodents and in humans (Mennenga et al., 2014; Milad and Quirk, 2012). See Stephan et al., 2019 for a review of other behaviors that can be translated across different species.

Conclusion.

Here, we have outlined the most basic and essential methodological considerations for designing experiments on HC in female rats. We present the pharmacology of HC (Tables 1 and 2) which should help in designing future experiments. In addition, we have provided an overview of past research (Table 3) and a framework for designing future experiments. What is clear is that there is no one perfect way to model HC in female rats. The elements of each model (vis. dose, ROA, exposure time/timing, and controls) will always depend on what level of analysis (Fig 4) is being examined. It is hoped that this review will help further propagate research on HC in rodents.

Preface to Chapter 3.

In chapter 2, we presented a model of HC that was designed in order to investigate the effects of exogenous hormones like those used in HC on memory bias. The aim of chapter 3 was therefore to use our model of HC in female rats to investigate memory bias. By so doing, it was demonstrated that the exogenous hormones contained in hormonal contraceptives also influence memory bias in female rats.

Chapter 3: Combined effects of the contraceptive hormones, ethinyl estradiol and levonorgestrel, on spatial navigation in gonadally intact female rats.

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Abstract

During spatial navigation rats can rely on hippocampus-mediated place memory or striatum-mediated response memory. Ovarian hormones bias whether females use place or response memory to reach a reward. Here, we investigated the impact of the contraceptive hormones, ethinyl estradiol (EE) and levonorgestrel (LNG), on memory bias. A total of 63 gonadally-intact female rats were treated with either 10μg/kg of EE alone, 20μg/kg of LNG alone, both 10μg/kg of EE and 20μg/kg of LNG together, or a sesame oil injection with 5% ethanol as a vehicle control. Rats in the control condition were tested during the diestrus phase of the estrous cycle in order to control for the low circulating levels of gonadotropin and ovarian hormones that occur with oral contraceptive administration. Rats treated with LNG alone had a bias towards to the use of place memory compared to diestrus phase control rats. This bias was not observed if LNG was administered in combination with EE. Rats treated with EE or EE+LNG did not have a statistically significant difference in memory bias compared to rats in the control group. These data show that

synthetic hormones contained in oral contraceptives administered to female rats influence which cognitive strategy is predominantly used during navigation.
Keywords: ethinyl estradiol; levonorgestrel; cognitive strategies; hippocampus; dorsal striatum; luteinizing hormone.

Introduction

Rodent navigation involves at least two distinct memory systems that rely on independent brain structures (Packard et al., 1989; Packard and McGaugh, 1992). *Place* memory is mediated by the hippocampus (HPC), and *response* memory is mediated by the dorsal striatum (DS; Packard and McGaugh, 1996). With place memory, rats access a "cognitive map" and navigate based on spatial cues in their environment (Tolman et al., 1946). In contrast, response memory refers to the repetition of well-practiced motor patterns and is characterized by being habitual, procedural, or automatic (Brake and Lacasse, 2018; Goodman, 2021). Using the dual-solution task, a variation of the plus-maze, one can distinguish between the use of place and response memory during navigation (Blodgett and McCutchan, 1948; Restle, 1957; Tolman et al., 1946).

It is by now well-established that when female rats are tested in the dual-solution task, the use of place or response memory largely depends on their ovarian hormone levels (Almey et al., 2014; Gomez-Perales and Brake, 2022.; Hussain et al., 2016; Korol, 2004; Korol and Kolo, 2002; Lacasse et al., 2022b; Quinlan et al., 2008, 2013). Among the estrogens (E), 17β-estradiol (E2) is the most potent and abundant naturally circulating estrogen in women and female rats (Hiroi et al., 2016; Nazari and Suja, 2016). Female rats use response memory more frequently when E2 levels are low. When E2 levels are high but within natural physiological ranges, however, they are biased towards the use of place memory (Hussain et al., 2016; Korol et al., 2004; Korol and Kolo, 2002; Lacasse et al., 2022b; Quinlan et al., 2008, 2013). When administered in conjunction with high levels of E2, progesterone (P) reverts the predominant use of place memory and instead promotes the predominant use of response memory (Korol and Pisani, 2015; Lacasse et al., 2022b). E2 infused into the HPC enhances place memory, whereas E2 infused into the DS impairs response memory (Korol and Wang, 2018; Zurkovsky et al., 2011, 2006). Together, these findings suggest that memory systems mediated by unique brain structures respond differently to ovarian hormones such as E2 and P. We have defined the concept that there is a bias to predominantly engage one memory system over another depending on circulating hormone levels in females as *memory bias* (Gomez-Perales and Brake., 2022.; Hussain et al., 2014; Hussain et al., 2016; Quinlan et al., 2013).Ovarian hormones have been shown to affect memory bias in both female rats and women (Almey et al., 2014; Brake and Lacasse, 2018; Hussain et al., 2016; Hussain et al., 2016; Hussain et al., 2013; Lacasse et al., 2022b; Quinlan et al., 2013, 2008)

No studies have examined the effects of synthetic estrogens or progestins such as those used in oral contraceptives (OC) on memory bias. Globally, about 151 million women take OC (United Nations, 2019). In the United States, 13% of the population take OC (Daniels, 2020). In Canada, ~16% of women between the ages of 15 and 49 take OC (Rotermann et al., 2015). It is not known whether synthetic hormones such as those contained in OC have the same effects on female cognition as ovarian hormones.

The mechanisms through which OC work and their pharmacological properties present a unique challenge. Any behavioral/cognitive effects associated with OC may be due either indirectly to the suppression of the hypothalamic-pituitary-gonadal (HPG) axis or to the direct activity of the synthetic hormones contained within OC themselves. Most OC work by inhibiting the HPG axis directly (Hilz, 2022; Lacasse et al., 2022a; Stanczyk et al., 2013). When hypothalamic release of gonadotropin releasing hormone (GnRH) is suppressed, pituitary release of gonadotropins like luteinizing hormone (LH) and follicle stimulating hormone are also significantly reduced (Rivera et al., 1999). It has been shown that LH mediates performance on HPC-mediated spatial tasks, though it has not been assessed in the dual-solution task used here. HPC-mediated spatial memory is improved by reducing LH levels or administering LH-antagonists (Bohm-Levine et al., 2020; Burnham et al., 2017; McConnell et al., 2012; Ziegler and Thornton, 2010), whereas increasing LH levels or administering LH-agonists impairs HPC-mediated spatial memory (Burnham et al., 2017; Casadesus et al., 2007). It is therefore possible that reducing LH levels could promote a place memory bias. Thus, the suppression of the HPG axis by administration of OC may influence memory bias. However, it remains unclear in which direction this effect might occur since reducing LH levels also reduces E, and low levels of E2 produce a response memory bias (Brake and Lacasse, 2018; Korol, 2004). It is important to investigate this as it may give insight into the effects of OC on brain structures such as the HPC and the DS which are known to be sensitive to changes in ovarian hormone levels.

A second possibility is that the synthetic hormones contained in OC such as ethinyl estradiol (EE) and levonorgestrel (LNG) may directly impact memory bias. EE is a synthetic estrogen used in many forms of OC (Dickey, 2021; Hampson, 2020). EE has 194% to 233% greater binding affinity for the estrogen receptor (ER) ERα compared to E2 (Escande et al., 2006; Jeyakumar et al., 2011). Memory bias may therefore be influenced by EE in a similar manner to what is observed when E2 levels are elevated. LNG is a 19 nor-testosterone derivative and has a modest binding affinity for progesterone receptors (PR) and a greater affinity for androgen receptors (AR; Kuhl, 2005; Schindler, 2015). Given its affinity for AR, LNG

is sometimes termed an "*androgenic*" progestin (Giatti et al., 2016) and has physiological effects similar to those of androgens (Lemus et al., 1992). In light of the fact that LNG binds PR as well as AR, it is not clear how it might affect place or response memory.

There have been only four studies that have examined the effects of EE and/or LNG on spatial learning in ovary-intact female rats of reproductive age. Nwakanma et al. (2021) showed that female rats treated daily with low doses of EE (0.2μg/kg) and LNG (0.43μg/kg) had shorter escape latencies in the Morris Water Maze (MWM) relative to vehicle treated controls. Two other studies have shown that rats treated with higher doses of EE (0.02mg or 0.03mg/day) and LNG (0.06mg or 0.125mg/day) did not perform differently in the MWM compared to vehicle-treated rats (Boi et al., 2022; Santoru et al., 2014). Moreover, Simone et al. (2015) found that rats treated with LNG (20μg/day) performed better in a novel context recognition task compared to female rats in the diestrus phase. However, performance on novel context recognition and novel place recognition tasks did not differ from naturally cycling diestrus phase controls when rats were treated with either EE alone (10μg or 30μg/day) or EE combined with LNG (10μg/20μg or 30μg/60μg/day). Based on these findings, EE and LNG do not seem to impact spatial learning except when given at lower doses (Nwakanma et al., 2021; Simone et al., 2015). It is therefore not clear how EE and LNG may influence performance in the dual-solution task based on these studies. Of note, while high levels of E2 tend to promote the use of place memory and low levels tend to promote response memory, levels of E2 do not predict maze learning (i.e., days to reach criterion; Korol and Pisani, 2015; Lacasse et al., 2022b). Thus, the synthetic hormones used in OC may influence which memory system is engaged during navigation without necessarily impacting spatial learning.

Rats treated with EE and LNG have suppressed levels of ovarian and gonadotropin hormones (Hilz, 2022; Lacasse et al., 2022a). Female rats in the diestrus phase of the estrous cycle have naturally low levels of both gonadotropin and ovarian hormones (Staley and Scharfman, 2005). Consequently, we used female rats in the diestrus phase as controls for the suppression of these hormones initiated by OC. Furthermore, rats in diestrus typically do not

show a particular bias for place or response memory (Korol et al., 2004). Here, we explore the effects of EE and LNG individually and in combination within the dual-solution task compared to female rats in the diestrus phase.

Methods

Test subjects. A total of 63 two-to-three-month-old Long-Evans female rats weighing ~240g were received from Charles River Laboratories (Kingston, New Jersey). Rats were pair-housed until food restriction began, upon food restriction all rats were housed individually in Ancare© plastic shoe-box cages (dimensions: 25.5 cm wide x 46.6 cm long x 21.6 cm high), under a 12 h reverse light-dark cycle (2000 to 0800). Before maze training began (habituation days 1 to 16), rats had *ad libitum* access to low-phytoestrogen rat chow (Teklad Global Rat Chow®) and water from an Ancare© plastic water bottle. Once training began (Day 16) rats were food restricted, maintaining their weights at 85% of free-feeding levels (~20-30g of chow daily; Toth and Gardiner, 2000). All procedures involving rats were performed in accordance with the guidelines established by the Canadian Council on Animal Care and approved by the Concordia University Animal Research Ethics Committee.

Hormone treatment. EE (Sigma-Aldrich, PHR1480) and LNG (Sigma-Aldrich, PHR1850) were suspended in 5% ethanol and sesame oil. Rats were assigned at random to one of four treatment conditions. Rats were treated with either 10µg/kg of EE alone (n=15), 20µg/kg of LNG alone (n=18), both 10µg/kg of EE and 20µg/kg of LNG together (n=14), or a sesame oil with 5% ethanol injection at the same volume (0.2-0.3µl) as the vehicle control (n=16). These doses were selected as they are reported to be the lowest dose needed to inhibit the rat estrous cycle (Andrews et al., 2002; Coelingh Bennink et al., 2008; Kumar, 2000; Lacasse et al., 2022a; Muhn et al., 1995). These doses are also consistent with another study that showed cognitive effects of EE and LNG (Simone et al., 2015). Injections were given

subcutaneously each morning between 0800 and 1000 for 21 consecutive days during maze training and testing. All rats remained gonadally intact for the duration of the experiment.

Estrous cycle monitoring. Just prior to treatment injections, samples from the vaginal epithelium were collected for purposes of estrous cycle monitoring (every 1-2 days). The purpose of monitoring vaginal cytology for the initial two weeks of maze training was to confirm that rats exposed to EE, LNG, or EE+LNG were not cycling, and that vehicle controls continued to show a regular 4-to-5-day estrous cycle. To collect vaginal line cells, 0.1 mL of deionized water was pipetted and retrieved from inside the vaginal canal and placed on a microscope slide. Slides were analyzed with a light microscope in order to characterize estrous cycle phases according to Westwood (2008). In the final week of training, samples were collected daily to confirm a state of acyclicity in hormone-treated rats and on test day, a state of diestrus in the control condition. Rats in the control condition were left to cycle freely over the 21 days of training. Free-cycling rats were only tested during the diestrus phase. In three cases, they were not in diestrus on the planned day of testing. In these cases, rats were tested within 24-48 hours once diestrus was confirmed. These rats were not given any additional training during these intervening days but were handled by experimenters and still given vehicle injections in the mornings in order to keep all other treatments consistent. It was also confirmed that these rats still reached testing criterion on the day they were tested.

Maze apparatus. Training and testing were carried out within a gray Plexiglas T-maze which was placed on a table 1m above the floor. The T-maze comprises gray walls (28cm high), a stainless-steel grid floor, transparent Plexiglas ceiling panels, a start arm (75cm long), and two target arms (each 75cm long) both positioned at a 90° angle to the start arm (Fig 1b). The start arm contained a sliding door which obstructs the first half of the arm, creating a start chamber. The start chamber included two transparent wall panels, which ensured that the rat could easily see the spatial cues in the environment from the starting position. The transparent ceiling panels

enabled rats to navigate according to spatial cues which were hung on the walls around the maze.

The target arm contained a small stainless-steel cup containing ¼ of a single unit of Kellogg's Froot Loops™ at its end. Throughout the entire maze, new crushed Kellogg's Froot Loops™ were placed below the grid floor daily in order to avoid the use of odor cues during habituation, training, and testing. Two additional sliding doors separated the target arms from the choice point of the start arm and could be closed to prevent the rats from leaving their chosen target arm once they were inside. An additional arm (identical in dimensions to the start arm) was added to the T-maze to form a plus-shape maze. A sliding door closed off access to this fourth arm of the maze at all times, except during probe testing. The T-maze was situated in a room dimly lit with overhead red lamps, a lamp facing the ceiling (40 W light bulb). Experimenters stood in a specified region located two feet from the plus-maze, serving as a spatial cue. Other spatial cues included cupboards and colorful posters hung overhead on the walls.

Maze training/testing. Upon arrival, rats were given a full day to acclimate to a new environment. Each rat was handled for 10-15 min daily for five days. Six days after arrival, rats were placed within the maze apparatus (with the sliding doors removed) for 5 min daily, for 10 consecutive days for maze habituation. This extended maze habituation period was intended to minimize stress and anxiety when in the maze. After sixteen days, rats were single housed, food restricted, and maze acquisition training began (Fig 1a).

Although the maze is shaped as a plus (Fig 1b), only three arms are ever made available to the rat during habituation, training, and testing. Only the arm which the rat was assigned to was baited, the other was left with an empty food bowl. All rats were randomly assigned and counter-balanced to be trained to turn into either the left or right arm. For 21 consecutive days, each rat was given 10 choice-trials where they could either enter the left or right arm of the maze. For each choice-trial, the rat was placed into the start chamber. Upon opening the sliding door of the start arm the choice trial began. The trial ended when the rat had

entered (all four paws) into either the left or right arm. Once a rat entered either arm a sliding door closed behind it, separating the rat into the respective arm it entered. The rat was then given an additional 20-30 sec either to retrieve its food reward or to examine its empty food bowl, depending on whether or not it turned into the baited arm.

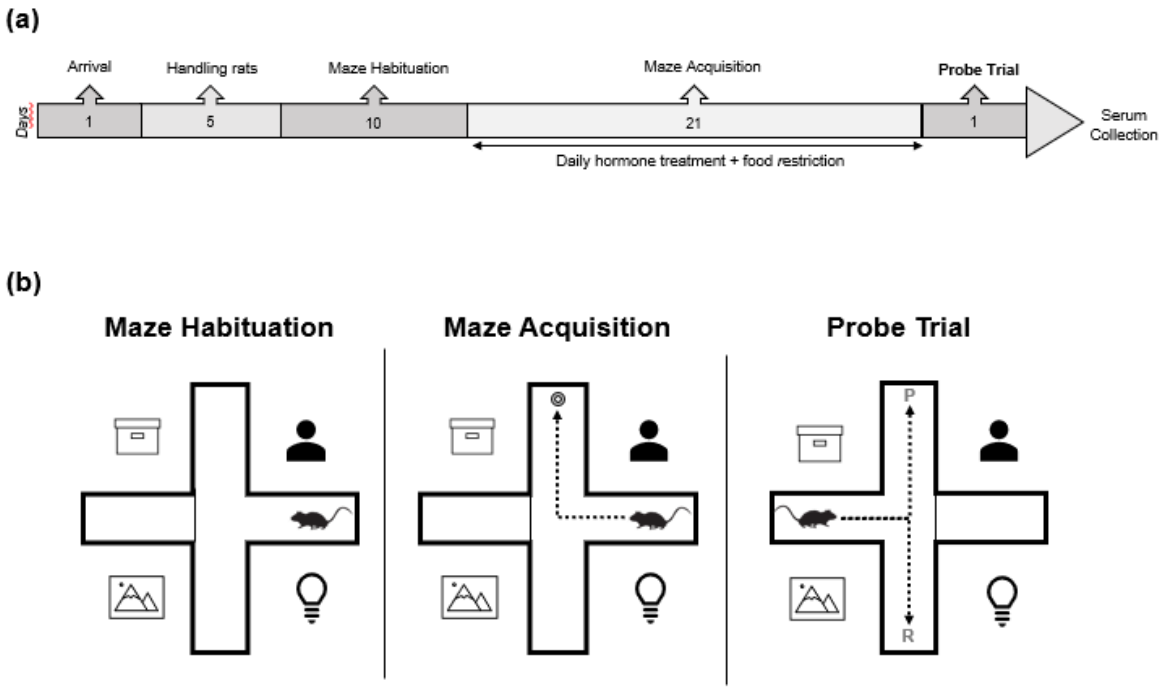

Figure 1. (a) A visual timeline of the protocol used for this experiment. (b) Illustration of the dual-solution plus-maze task set up during each stage of the experiment. During maze habituation, there are no food rewards within the maze and no target arm for the rat. During maze acquisition, rats are consistently trained to retrieve a food reward from the same goal arm, in this case by turning right. On the probe trial, the rat is then placed 180 degrees opposite to its original start position. If the rat turned into the *same arm location* that it was trained it was recorded to be using place memory (P). If the rat turned in the *same direction* that it was trained, it was recorded to be using response memory (R). The images around the maze represent the spatial cues within the environment relative to the goal arm and the rats starting position.

Trial latency was recorded as the amount of time (sec) between the opening of the start arm and the closing of either the left or right arm sliding door behind the rat. The number of times the rat entered each arm was also recorded. Testing criterion was achieved when a rat

turned into the correct arm 8/10 times for three consecutive days. Days to reach criterion was recorded as the number of training days needed before testing criterion was achieved.

On day 21 of maze training, rats were tested using a dual-solution paradigm (Fig 1b). Rats were placed into the start chamber 180 degrees opposite to the start arm in which they were trained. For the probe trial, we assessed whether the rat turned towards the same arm location it had been trained (e.g., turning towards the same environmental/spatial cues), or whether it maintained the same turn direction (e.g., turning in the same direction it was trained). If the rat turned into the *same arm location* that it was trained it was recorded to be using place memory. If the rat turned in the *same direction* that it was trained, it was recorded to be using response memory.

Training and testing took place between 1200 and 1700. The maze was regularly cleaned after training each day and new crushed Froot Loops were scattered below the grid floor throughout the entire maze to mask scent cues. A single experimenter carried out the injections and cycle-tracking, and a separate group of experimenters carried out the behavioral training/testing. All experimenters who participated in regular handling, behavioral training, and feeding of the rats were blind to hormonal treatment conditions.

Luteinizing Hormone Assays. In order to confirm suppression of the hypothalamic-pituitary-gonadal axis, LH levels were assayed at the conclusion of the experiment. Within 15 minutes of behavioral testing rats were euthanized, trunk blood was collected and centrifuged within one hour. Serum was extracted and stored at -20 ◦C. No anticoagulants were used. Serum LH concentration (ng/mL) was analyzed via a commercially available enzyme-linked immunosorbent assay (ELISA) from Abnova (Catalog number: KA2332). This kit reports a sensitivity of 0.50ng/mL and a limit of detection (L.O.D.) of 0.5ng/mL.

Statistical Analysis. All statistical analyses were performed using IBM® SPSS® Statistics software. A repeated measures analysis-of-variance (ANOVA) test was performed to compare groups on mean trial latency across 21 days of training. A one-way ANOVA was

performed to compare groups on mean number of days to reach criterion. To test memory bias, the data were categorical and thus, nonparametric statistics were used. Three independent 2x2 chi-square tests were conducted to determine whether any treatment condition showed a significant bias toward place or response compared to what was observed within the vehicle control condition. In lieu of effect sizes, odds ratios were calculated using Microsoft Excel with the Analysis ToolPak for statistical analysis add-on for each treatment condition in order to determine if there was an association between treatment and navigation strategy used.

Results

Confirmation of acyclicity. Converging lines of evidence were used to confirm that the rats given either EE, LNG, or EE+LNG were not cycling. Rats administered EE, LNG, or EE+LNG never showed vaginal cytology that indicated a state of proestrus (clusters of predominantly round nucleated epithelial cells) nor estrus (clusters of cornified enucleated epithelial cells). In all cases where EE, LNG, or EE+LNG were administered rats remained in a persistent state of metestrus/diestrus as indicated by a relatively even ratio of nucleated and cornified epithelial cells, as well as leukocytes. It should also be noted that rats that received either EE alone or EE+LNG had a greater abundance of nucleated epithelial cells compared to rats administered LNG alone. The presence of vaginal mucus was observed in all rats in this study. Rats in the vehicle control condition had regular 4-to-5-day estrous cycles throughout the entirety of the experiment.

Serum collected from female rats treated with EE (*n*=13), LNG alone (*n*=13), EE+LNG (*n*=12) had levels of LH that fell below the limits of detection (0.50ng/mL) of the assay. Female rats in the diestrus phase (*n*=12) had a mean serum LH concentration of 4.09ng/mL ± 2.13 (range detected 0.54-25.21ng/mL). Intra-assay reliability was 6.05%CV and inter-assay reliability was 7.68%CV. One sample from the LNG condition was removed from all behavioral

analyses as it had circulating LH levels three standard deviations higher than the rest of the rats in the same treatment condition.

Days to criterion. A subset of 10 rats did not reach criterion by test day and were removed from the analyses. As a result, only 52 of 63 rats were included in the final analysis: EE (*n*=14), LNG (*n*=12), EE+LNG (*n*=14), diestrus (*n*=12). Although anecdotal, rats who did not reach criterion after 21 days did not show any obvious behaviors (i.e., freezing, grooming) that were explicitly different from those who managed to reach criterion. There was still a decrease in trial latency across the 21 days of training as was observed in other rats. In these 10 cases, rats did not consistently (≥8/10) enter the correct arm for 3 consecutive days at any point during the 21 days of training. Most rats not reaching criteria were in the LNG or vehicle condition (Fig 2a). In terms of days to reach criterion, a one-way ANOVA revealed that there was no statistically significant difference in days to reach criteria between groups F (3,48) = 2.20, p = 0.09 (Fig 2b).

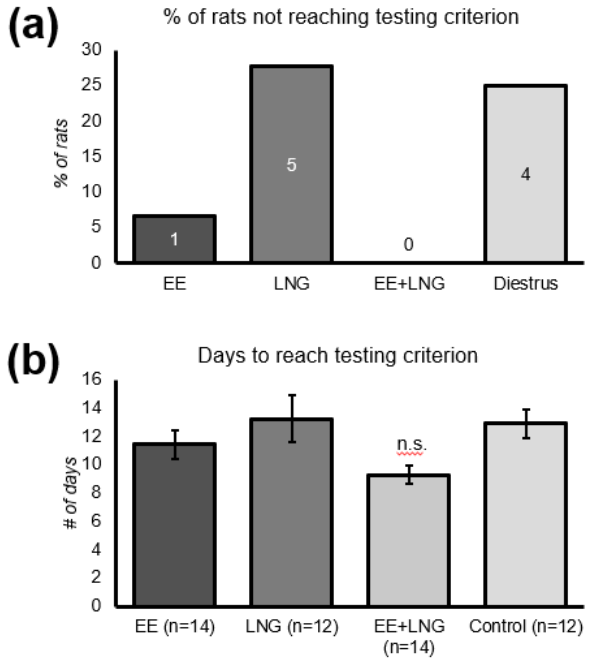

Figure 2. (a) percentage of rats in each treatment condition that did not reach the testing criterion by test day. Numbers in bars represent the actual number of rats not reaching criterion; (b) the mean number of days to reach criterion for each treatment condition. Error bars represent standard error of the mean.

Trial Latency. A repeated measures ANOVA revealed a statistically significant effect of trial such that mean trial latencies decreased across the 21 days of training across all treatment conditions F (20, 980) =11.927, p = <0.01. Although mean trial latencies decreased over training, there was no effect of treatment condition on mean trial latency F (3, 49) =.347, p = 0.791 and no trial x treatment interaction F (60, 980) = 1.011, p = 0.455.

Memory bias. Chi-square analysis revealed that there were no statistically significant differences in use of place or response memory in the EE group (χ^2 (1) = 2.476, p = 0.116) or the EE+LNG group (χ^2 (1) = 0.69, p = 0.793) relative to what was observed in the diestrus controls. However, there was a statistically significant difference between the LNG treated rats and the diestrus controls; χ^2 (1) = 6.171, p = 0.013 such that rats treated with LNG showed a statistically significant bias toward using place memory.

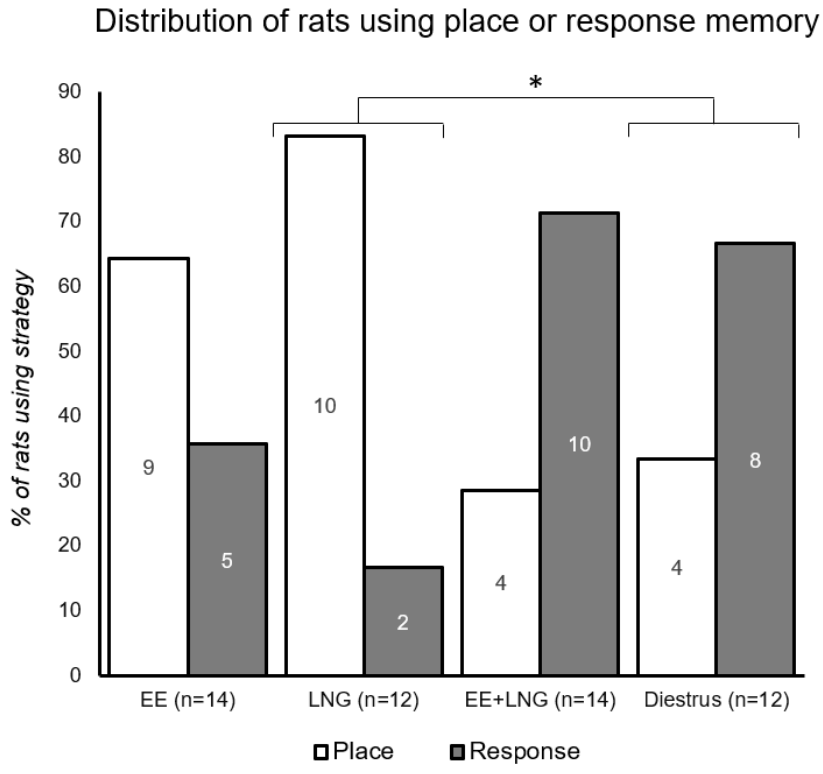

Figure 3. The distribution of rats using place and response memory within each treatment condition. Compared to female rats in the diestrus phase administered LNG alone had a place memory bias. No other treatment condition produced a statistically significant difference in bias for using place or response memory compared to female rats in diestrus. Numbers in bars represent the actual number of rats using place or response memory.

Analysis of odds ratios showed that the probability of using place memory was 10x greater in rats treated with LNG compared to diestrus controls (Table 1). The odds ratios values we observed are consistent with values obtained from similar studies administering E2 or P (Almey et al., 2014; Hussain et al., 2016a, 2013; Lacasse et al., 2022b; Quinlan et al., 2013, 2008).

Table 1.

Odds ratios for use of place memory in treatment groups relative to controls.

Treatment group	Place | Response
EE (*n*=14)	**3.15** [16.31 / 0.61]
LNG (*n*=12)	**10.00** [69.26 / 1.44]
EE+LNG (*n*=14)	**0.80** [4.24 / 0.15]
Diestrus (*n*=12)	**1**

Note. Table 1 shows the odds ratios for the use of place memory in treatment groups relative to female rats in the diestrus phase. Any value that is <1 is less probable and any value >1 is more probable. Numbers in square brackets represent [upper / lower] confidence intervals (C.I.). If the C.I. captures 1 there would be no difference between groups.

Body weight. There were body weight changes observed in all groups from day 1 to day 21 of training. Rats treated with EE had an average weight of 244.6g ± 3.81 on day 1 and 216.93g ± 4.09 on day 21 (27% weight reduction). Rats treated with LNG had an average weight of 252g ± 2.14 on day 1 and 278.88g ± 2.86 on day 21 (27% weight gain). Rats treated with EE+LNG had an average weight of 247.21g ± 3.84 on day 1 and 228.21g ± 4.95 on day 21 (19% weight

reduction). Finally, female rats in the control group had an average weight of 240.6g ± 3.16 on day 1 and 256.73g ± 4.23 on day 21 (14% weight gain).

Discussion

It was anticipated that rats treated with EE, LNG, or EE+LNG would show a different relative use of place or response memory compared to female rats in the diestrus phase. Although rats treated with LNG showed differences from females in diestrus, this was not the case for rats treated with EE or EE+LNG. The difference in relative use of place and response memory between rats treated with LNG and diestrus phase controls was statistically significant. In comparison to controls, rats treated with LNG were 10x more likely to use place memory. We cannot identify the mechanism by which LNG promotes place memory until further experiments are conducted, since this study measured behavior only. There have been no studies that have assessed the role of P without E2, or of androgens on memory bias in female rats. Due to this, there is no point of comparison for how PR or AR activity may influence memory bias. Nevertheless, our findings are in line with at least one study which demonstrated that administering the same dose of LNG improved novel context recognition compared to diestrus phase controls which was not observed with EE alone or when EE and LNG were given in combination (Simone et al., 2015).

The observations of a place memory bias in rats treated with LNG could be similar to those observed in gonadectomized (GDX) male rats treated with androgens like T and dihydrotestosterone (DHT; Lemus et al., 1992). For example, T promotes place memory in GDX male rats in a dose-dependent manner (Spritzer et al., 2013; Wagner et al., 2018; Zhang et al., 2020). In addition, T and DHT enhance spatial memory performance in GDX male rats on other HPC-mediated tasks (Jacome et al., 2016; McConnell et al., 2012; Moghadami et al., 2016). Yet, we cannot necessarily generalize observations from male rats to female rats because they perform differently in the dual-solution task (Brake and Lacasse, 2018; Korol and Wang, 2018).

Androgens such as DHT, dehydroepiandrosterone (DHEA), and androstenedione improve HPC-mediated object placement memory in ovariectomized (OVX) female rats (Luine et al., 2022). Similarly, OVX female rats given DHEA immediately after training show increased spatial learning in the MWM after 24 hours (Frye and Lacey, 1999). In a study conducted by Roof and Havens (1992), neonatal female rats injected with T propionate (300μg) on post-natal days 3 and 5 exhibited improved spatial learning when later tested at reproductive age. Together, these findings suggest that androgens can indeed affect performance on HPC-mediated spatial tasks in female rats. In fact, AR immunoreactivity has been observed throughout the female rat HPC (Xiao and Jordan, 2002). Hence, LNG's affinity for AR may explain the effect we observed.

Human studies have shown that women with short CAG repeats, which increase AR sensitivity, perform better on spatial navigation tasks than women with longer CAG repeats, who are less sensitive to androgens (Nowak et al., 2014). Thus, women's performance on spatial tasks appears to be at least partially influenced by androgens. Structural brain imaging studies have also shown that women taking OC have altered grey and white matter volume both in the HPC and the DS when compared to those not taking OC (Pletzer et al., 2019a; Sharma et al., 2020c). Women taking androgenic forms of OC consistently perform better on spatial navigation tasks compared to women who are not taking OC (Bernal et al., 2020; Bianchini et al., 2018; Gurvich et al., 2020; Patel et al., 2022; Piber et al., 2018). However, since OC contain both a synthetic estrogen and a progestin, the results observed in women who take OC do not explain why EE+LNG treatment did not produce the same place memory bias as did LNG treatment. Though rats treated with LNG differed from controls in memory bias, treatments did not have any effect on days to reach criterion or trial latency over the 21-day training period. This suggests that LNG may alter memory bias while having no impact on learning the spatial environment.

It is not clear from this study how LNG might impact memory bias via action in the DS as no studies have examined androgens in this context. In previous studies, elevated levels of E2

reduced the likelihood of female rats using response memory (Almey et al., 2014; Hussain et al., 2013; Hussain et al., 2016; Lacasse et al., 2022b; Quinlan et al., 2008, 2013). Likewise, intra-striatal infusions of E2 impair response learning within two hours of administration (Zurkovsky et al., 2011). HPC-mediated place memory is also promoted when the DS is lesioned or functionally inactivated (Packard et al., 1989; Packard and McGaugh, 1996). LNG may promote place memory through actions similar to those of E2 in the striatum. However, we may not be able to apply these findings to our observations since this speculation is based on findings with E2.

When administered alongside EE, LNG no longer promoted a place memory bias as was seen in rats treated with LNG alone. This finding is consistent with previous work demonstrating that when 10μg of either E2 or estradiol benzoate (EB) are administered, female rats show a place memory bias. Yet, if the same doses of E2 or EB plus an injection of P are administered, rats show a response memory bias (Korol and Pisani, 2015; Lacasse et al., 2022b). Thus, in combination E2 and P do not promote place memory which is comparable to what was observed with EE+LNG. This finding could be due to the fact that E2 has been shown to induce PR within the HPC (Guerra-Araiza et al., 2003; Parsons et al., 1982). Perhaps administering EE increases hippocampal PR, altering LNG's effects compared to administering LNG alone. This needs to be investigated in future studies. Still, the important observation is that rats administered LNG alone exhibited a clear bias toward using place memory, while only a small number of rats administered EE+LNG used place memory. Thus, the action of LNG on memory bias is different depending on whether it is administered in the presence of EE.

There was no statistically significant difference between rats treated with EE alone and diestrus phase controls in the use of place or response memory. This finding runs counter to past literature which suggests that elevated E2 promotes a place memory bias (Almey et al., 2014; Hussain et al., 2013; Hussain et al., 2016; Korol et al., 2004; Korol and Kolo, 2002; Lacasse et al., 2022b; Quinlan et al., 2008, 2013), and also improves performance on spatial

tasks mediated by the HPC (Daniel et al., 1997; Frick, 2015; Frick and Kim, 2018b; Frye et al., 2007; Patel et al., 2022; Taxier et al., 2020). Although, Mennenga et al. (2015) demonstrated that EE dose dependently impaired performance on spatial tasks such as the Water Radial Arm Maze and the MWM. However, the rats tested in that study were OVX and therefore may not necessarily relate to our observations testing ovary-intact females. It should be noted that in both conditions where treatment contained EE, all rats reached testing criterion. This may suggest that EE improved rats' ability to reach testing criterion compared to those treated with LNG and those in the control group. Additionally, one previous study did not show any memory bias in female rats in the diestrus phase (Korol et al., 2004a). Thus, our findings in diestrus phase females are consistent with that previous study.

Another possible explanation for altered memory bias when administering synthetic hormones like EE or LNG is the suppression of gonadotropins such as LH. Studies have suggested that lowering LH levels may promote HPC-mediated spatial memory (Bohm-Levine et al., 2020; Burnham et al., 2017; McConnell et al., 2012; Ziegler and Thornton, 2010). The levels of LH in rats treated with EE, LNG, and EE+LNG were below the detection limits of our assay. All three treatments significantly decreased LH levels, but only LNG significantly promoted HPC-mediated place memory. Therefore, suppression of LH alone cannot explain the place memory bias observed in rats treated with LNG, since lowered LH was also observed in the other treatment groups.

A limitation of this study is that the probe trial simply cannot account for other cognitive processes that may influence how the rat behaves i.e., attentional processes, decision making. The design of this experiment also limits our ability to parse whether the effects we observed are due to chronic treatment over 21 days with EE and LNG, or whether the same effects would have been observed with a single acute injection on test day. It is possible that a single injection of EE, LNG, or EE+LNG could have been sufficient to alter place and response memory in the same way. Acute administration of EE and LNG has little translational relevance to the use of

OC in humans. However, acute vs. chronic injection of these hormones may offer some insight into the underlying mechanisms for the behaviors observed.

Conclusion.

The synthetic hormones used in OC impact memory bias in female rats. Specifically, LNG administered alone promotes a place memory bias, an effect that is not observed when LNG is paired with EE. Moreover, we observed no particular bias to use place or response memory in rats treated with EE alone or diestrus phase controls. Thus, the impact of LNG on memory bias will vary depending on whether LNG is given alone or in combination with EE. This may have implications for understanding the cognitive effects of different forms of hormonal contraceptives which either contain both EE and a progestin, or progestin-only forms. More research is needed to uncover the mechanisms underpinning the impact of LNG on memory bias in female rats, and how EE may alter that.

Preface to Chapter 4.

Having found that both endogenous and exogenous hormones impact memory bias during spatial navigation in female rats in chapters 1 and 3, we then turned our attention to humans. The final aim of this thesis was therefore to examine the effects of both endogenous and exogenous gonadal hormones on memory bias in humans. Thus, in Chapter 4 we demonstrate that both endogenous and exogenous gonadal hormones can impact memory bias and navigation performance in humans. These findings highlight the importance of considering endogenous and exogenous hormones when studying spatial navigation.

Chapter 4: Biological sex, gonadal hormones, and oral contraceptives impact spatial navigation in a 3-dimensional virtual Hex maze task.

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Abstract

A sex difference in spatial navigation has been well-established, but the role of gonadal hormones remains unclear. Endogenous hormones such as 17β-estradiol, progesterone, and testosterone as well as exogenous hormones such those contained in oral contraceptives may play a role in this sex difference. Naturally-cycling women (*n*=57), women taking oral contraceptives (*n*=51), and men (*n*=53) were recruited, and salivary levels of gonadal hormones were assessed with liquid chromatography-mass-spectrometry. Participants were tested within a virtual Hex maze task which assesses whether they use allocentric or egocentric strategies during navigation. It also measures latency as a marker of task performance. Men completed the task more quickly than naturally-cycling women but not women taking oral contraceptives. This sex difference was only observed when naturally-cycling women had low levels of 17β-estradiol and progesterone. Salivary progesterone levels were positively correlated with the use of egocentric strategies, and negatively correlated with the use of allocentric strategies. Salivary testosterone was also negatively correlated with latency to complete the task. Lastly, the type of oral contraceptive that was being taken influenced task latency. Taken together, these findings outline the importance of considering endogenous and exogenous hormones when studying sex differences in spatial navigation.

Keywords: ethinyl estradiol; oral contraceptives; cognitive strategies; spatial navigation; sex differences; testosterone; progesterone.

Introduction

Men and women perform differently on various cognitive tasks (Andreano and Cahill, 2009). Some of the most well-established cognitive sex differences in humans are found in spatial abilities (Levine et al., 2016; Linn and Petersen, 1985). There is generally an advantage for men in terms of spatial abilities, specifically on mental rotation tasks (Linn and Petersen, 1985; Voyer et al., 1995) and spatial navigation tasks (Brake and Lacasse, 2018), but not in terms of object location memory (see Voyer et al., 2007). While sex differences in spatial abilities are well established, the moderating role of gonadal hormones in this effect remains unclear, particularly in human studies of spatial navigation.

A well-established distinction exists between two cognitive strategies that can be applied during spatial navigation (Bohbot et al., 2012; McDonald and White, 1994; Packard and McGaugh, 1992).Using an allocentric or place strategy, the navigator forms a 'cognitive map' of the environment and navigates through it by using cardinal directions (*e.g.,* "head north, then west") or Euclidean-terms (*e.g.,* walk 500m, or travel 20 miles; Epstein et al., 2017; Harris et al., 2022). The allocentric frame of reference is independent of the navigators perspective, as their global understanding of the environment is not altered relative to their position (Spriggs et al., 2018). Human brain imaging studies have linked allocentric strategies to hippocampal activity (Bohbot et al., 2004; Maguire et al., 2003). Egocentric strategies, on the other hand, use well-practiced actions based on a procedural motion (*e.g.,* turning right, then left) or navigating according to a single cue or landmark in the environment (*e.g.,* heading straight for the red house). Egocentric strategies are therefore subdivided into '*response-based*' which has been associated primarily with activity in the striatum (Doeller et al., 2008; Iaria et al., 2003), or '*cue-based*' which is associated with activation across the parietal lobe (Blanch et al., 2004; Gramann et al., 2009; Lin et al., 2015; Plank et al., 2010). Egocentric strategies largely depend on the perspective of the navigator, and therefore behavioral responses are altered depending on their position in space (Spriggs et al., 2018).

Allocentric strategies are more frequently used by men during navigation than by women (Astur et al., 2016; Sandstrom et al., 1998; Spriggs et al., 2018). In contrast, women tend to rely primarily on egocentric strategies (Andersen et al., 2012; Piber et al., 2018; Sandstrom et al., 1998; Scheuringer and Pletzer, 2017). Men tend to learn maze environments and complete navigation tasks more quickly, and have greater accuracy during navigation (*i.e.,* retracing their steps less frequently; Astur et al., 2016; Daugherty et al., 2015; Gazova et al., 2013; Harris et al., 2022; Korthauer et al., 2017; Mueller et al., 2016; Nowak et al., 2014; Nowak and Moffat, 2011; Piber et al., 2018; Scheuringer and Pletzer, 2017; van Gerven et al., 2012).To be clear, not all studies have observed a sex differences in performance on navigation tasks (Bernal et al., 2020; Mueller et al., 2016; Rodgers et al., 2012; Sneider et al., 2015), but these inconsistencies may be due to different tasks being used and participants being tested at different ages.

The role of gonadal hormones in spatial navigation has been demonstrated clearly in rodent studies. In male rats, testosterone (T) has been shown to dose-dependently promote ‘place memory’ (Spritzer et al., 2013; Wagner et al., 2018; Zhang et al., 2020), which is analogous to allocentric strategies in humans. It has also been shown that levels of ovarian hormones, such as 17β-estradiol (E2) and progesterone (P), alter which strategy predominates in female rats. For example, when levels of E2 are low rats predominantly use ‘response memory’ (analogous to egocentric strategies). When levels of E2 are high rats predominantly use ‘place memory’ (Almey et al., 2014; Hussain et al., 2016a; Korol, 2004; Korol and Kolo, 2002; Quinlan et al., 2013, 2008). Rats that are administered P in combination with high E2 revert to predominantly utilizing 'response memory' (Korol and Pisani, 2015; Lacasse et al., 2022b). However, so far results of experiments on rodents have not readily translated to human studies.

The role of T in spatial navigation is not clear. In men, spatial navigation performance is either improved by T (Choi and Silverman, 2002; Driscoll et al., 2005) or unaffected by T

(Burkitt et al., 2007). Past studies have shown no connection between women's circulating levels of T and whether they primarily use allocentric or egocentric strategies (Hussain et al., 2016b; Scheuringer and Pletzer, 2017). In addition, elevated T was found to either improve (Burkitt et al., 2007) or not have any effect on women's navigation performance (Choi and Silverman, 2002; Driscoll et al., 2005; Scheuringer and Pletzer, 2017). The results of these studies do not clearly indicate whether T levels affect navigation strategies or performance in men or women.

Spatial navigation performance may also be impacted by ovarian hormones during the menstrual cycle (MC). For example, Hussain et al. (2016) found women used egocentric strategies in 3-D virtual radial-arm maze tasks during the early follicular (low E2, low P) and ovulatory phases (high E2, low P). During the mid-luteal phase (moderate E2, high P), women tended to use an allocentric strategy (Hussain et al., 2016). According to Scheuringer and Pletzer (2017), increases in P are associated with increased use of egocentric strategies, similar to what has been reported in female rats (Korol and Pisani, 2015; Lacasse et al., 2022). However, when participants were provided with egocentric instructions (e.g., head straight towards the school) in a 2-D navigation task, the MC phase was not associated with performance (Bernal et al., 2020; Scheuringer and Pletzer, 2017). Based on these results, cognitive strategies used during navigation are sensitive to changes in ovarian hormone levels, but the direction of these effects is not entirely clear.

Compared to naturally-cycling (NC) women, women who take oral contraceptives (OC) have a unique hormonal milieu. With OC, synthetic analogues of ovarian hormones are introduced into circulation while endogenous ovarian hormones are suppressed (Rivera et al., 1999; Stanczyk et al., 2013). Studies have shown that women taking OC differ from NC women on a variety of cognitive tasks (Beltz, 2022; Griksiene et al., 2022). It is important to note that these differences are not uniform across all women taking OC, and depend on the pharmacological properties of the particular formulation of OC (Beltz et al., 2015; Hampson,

2020). For example, OC differ from one another in terms of their dose of ethinyl estradiol (EE) and vary in terms of their estrogenic potency depending on which progestin is also included in the formulation (Dickey, 2021). A number of OC contain synthetic progestins derived from 19-nor testosterone (Kuhl, 2005). Besides binding to progesterone receptors (PR) with modest affinity, these progestins also bind to androgen receptors (AR) and exert androgenic effects (Darney, 1995; Giatti et al., 2016). Progestins derived from synthetic forms of P, such as 17-hydroxyprogesterone and 19 norprogesterone, have greater affinities for PR and very low affinities for AR (Sitruk-Ware, 2006). Accordingly, these progestins possess 'anti-androgenic' properties, or properties that oppose those of androgens.

Studies that evaluate navigation performance consistently show that those who take OC perform better than NC women (Bernal et al., 2020; Bianchini et al., 2018; Gurvich et al., 2020; Patel et al., 2022; Piber et al., 2018). Studies examining only androgenic forms of OC show improved performance on navigation tasks when compared to NC women (Bianchini et al., 2018) and better maze recall compared to anti-androgenic forms (Gurvich et al., 2020). The relative use of allocentric versus egocentric strategies during navigation has not been studied yet in relation to OC. In this study, we examine how sex, MC phase, and OC affect navigation strategies and performance in a virtual Hex maze. This computer-based maze dissociates between the use of allocentric and egocentric strategies, separating the latter into ‘response-based’ and ‘cue-based’ (Spriggs et al., 2018). Using the Hex maze, the latency to complete the task across trials can also be used to measure navigation performance (Spriggs et al., 2018). As a result, we are able to analyze if levels of endogenous hormones relate to performance.

Past studies examining MC effects and OC have either not measured gonadal hormone levels (Gurvich et al., 2020; Piber et al., 2018) or used enzyme-linked immunoassays (ELISA) to measure them (Bernal et al., 2020; Hussain et al., 2016b; Patel et al., 2022). There have been recent criticisms of the reliability and validity of ELISAs for measuring salivary steroid hormone concentrations compared with liquid chromatography-mass spectrometry (LC/MS; Arslan et al.,

2022; Gao et al., 2015; Rosner et al., 2013; Schultheiss et al., 2018). Thus, our study contributes to the current literature by using a navigation task that distinguishes between three (rather than two) navigation strategies, as well as using the current gold-standard for salivary hormone measurement. On the basis of the literature outlined above, we expected men to use allocentric strategies and NC women to use egocentric strategies during navigation. Additionally, it was hypothesized men would complete the maze with shorter latencies than women (NC and OC). As compared to NC women, it was expected that women taking 2nd generation OC (due to their heightened androgenic activity) would primarily use an allocentric strategy and have shorter total latencies to complete the maze, as would be expected in men.

Methods

Participants.

214 people were recruited as a convenience sample from the Concordia University undergraduate student population and the local community (Montréal, Québec, Canada). Social media and poster advertisements were used to recruit participants on the university campus and in the community. To determine initial eligibility, participants were contacted by email and asked to provide basic demographic information. Participants were English-speaking, free of neurological and psychiatric disorders, and not using drugs that might affect cognitive performance (*e.g.,* amphetamines, methylphenidate, or modafinil). Individuals with uncorrected visual impairments, anabolic steroids use, or endocrine-related disorders such as polycystic ovary syndrome were also excluded. The study took place between March 2019 and March 2020. A cash payment of $25.00 CAD, or two participation pool credits, was offered to volunteers. All study procedures involving study participants were approved by Concordia's Human Research Ethics Committee.

The initial sample consisted of 73 NC women, from which a total of 16 individuals were excluded from further analyses. Twelve women were excluded as they did not provide a saliva

sample for hormone quantification, and four were excluded due to having levels of E2/P that were greater than two standard deviations above the mean of the group. Many NC women had very low or undetectable levels of E2 and/or P, yet some NC women had elevated levels of P. This was likely due to women being tested at different points in their MC. On the basis of these differences, NC women were therefore subdivided to create two subgroups with distinct levels of salivary P. NC women were classified into the NC_{low} group (*n*=42) if their salivary P levels were lower than 10pg/mL and as NC_{high} (*n*=15) if their salivary P levels were higher than 10pg/mL. During the follicular phase, P levels are very low or unmeasurable (Hampson, 2020; Sundström-Poromaa et al., 2020). Thus, levels above 10pg/mL were used as an indication that NC women were entering or were in the luteal phase of the MC.

Our initial sample consisted of 84 individuals taking OC, from which a total of 33 individuals were excluded from further analyses. In order to maintain a homogeneous sample of people taking similar OC types, participants taking first generation (e.g., norethisterone, norethindrone acetate) (*n*=4), fourth generation (e.g., drospirenone, cyproterone acetate) (*n*=9), or who used hormonal intrauterine devices (*n*=7), vaginal rings (*NuvaRing*©; *n*=2), or patches (*Evra patch*©; *n*=1) were excluded from the sample. In addition, participants who did not sufficiently report information about their OC type were also excluded (*n*=10). Thus, the final analysis included only women taking OC with either second generation (e.g., levonorgestrel) or third generation progestins (e.g., desogestrel, norgestimate, gestodene). Second generation and third generation progestins are all derived from the synthetic 19-nor testosterone (Kuhl, 2005), which are defined as "*androgenic*" progestins (Darney, 1995; Giatti et al., 2016; Sitruk-Ware, 2006). Final analysis of the OC group comprised individuals taking 2^{nd}Gen (*n*=32) and 3^{rd}Gen (*n*=19) OC types only. From an initial sample of 57 men, 4 were excluded due to faulty recording of Hex maze data. Thus, this study included 161 participants divided into three groups: NC (*n*=57), OC (*n*=51) and M (*n*=53).

Materials.

Demographic questionnaire. We assessed demographic characteristics such as; age, weight, height, sex, gender, education, medical conditions, and video game experience. We used, the Raven's Advanced Progressive Matrices (APM) measure of fluid intelligence (Bors and Stokes, 1998). We assessed perceptual speed using the Identical Pictures task (Lindenberger et al., 1993).

Women who indicated that they were using hormonal contraceptives (HC) were asked to fill out an additional questionnaire related to their HC. They were asked to report the type of HC (pill, IUD, vaginal ring, patch, injection), length of use, and where they were in their OC cycle. Additionally, information from the packaging of the OC was requested, such as the brand, the EE dosage, the progestin dosage, and the number of pills in a packet. Moreover, women who indicated that they were naturally cycling were asked for information related to their current estimated cycle phase (e.g., average cycle length, number of days since the beginning and end of the previous cycle).

Hex maze. All spatial navigation training and testing was conducted in the virtual Hex Maze (Spriggs et al., 2018). The UnReal Development Kit (UDK® Epic Megagames) was used to create and run the maze task and the computer used to run it was a Windows 10 desktop computer running an Intel® CoreTM 3.20GHz chip with a 23" screen resolution 1680x1050 placed ~2 feet away from the participant. An Xbox 360-type controller with buttons programmed to allow only left, right, and forward movements (both joysticks were disabled) was used by participants to navigate within the virtual maze. The environment and dimensions of the virtual maze were identical to what was described by Spriggs et al. (2018).

Procedure.

This study had a cross-sectional design with testing between 09:00 and 17:00. The participant's written and informed consent was obtained upon arrival at the lab. Participants

were asked to complete initial questionnaires at a testing computer station. The additional questionnaire was provided to women regarding their menstrual cycle and use of hormonal contraceptives. Participants were instructed not to use their cell phones during the remainder of the study and to follow all task instructions. The experimenter read from a script during maze training and testing to instruct participants how to proceed.

Training. Three phases of training were conducted, including exploration, visible platform trials, and explicit probe trials. During the exploration phase, participants were virtually placed at the eastern end of the maze environment, outside the maze (in the grassy area visible in Fig 1). There was no time limit on how long participants could familiarize themselves with the controls, the controller itself, and the virtual environment. The participants explored the maze environment from the grassy area surrounding the circular maze (Fig 1). Participants were also instructed to look out all of the windows in order to familiarize themselves with the extra-maze environment. The exploration phase ended when the participant informed the experimenter they had learned enough about the environment and how to use the controller.

During training, four visible platform trials were included to facilitate learning how to maneuver towards visible platforms. Among the arms of the practice maze, 1/6 had a silver platform on the ground. Participants were virtually teleported to the end of 1/6 arms and instructed to find the visible platform as quickly as possible. Platforms were located, in order: 1) the center of the arena, 2) the far end of the arm in line with the starting arm, 3) the second arm on the right from the start arm, and 4) the first arm on the left from the start arm. Upon reaching the platform, there was an audible beep. Participants were told they could look around the environment as much as they wanted, but they could not navigate off the platform. Maze windows were removed during visible platform trials, masking the extra-maze environment. In addition, each arm's colored spheres were removed during these 4 trials. Following the visible platform trials, the computer monitor was briefly turned off and participants were asked to set down the controller to receive further instructions regarding the upcoming explicit probe trial.

Figure 1. Hex maze environment seen from above. There is a green "grassy" area surrounding the maze in which the participants are placed during the exploration phase. In addition to the rectangular windows that surround the maze, there is also a circular window above it. The extra-maze environment is visible through the windows. Each arm ends with a sphere, one of which is colored while the others are the same color as the maze floor. For participants using a cued strategy, the colored sphere serves as a cue.

In order to complete the training, participants were required to complete an explicit probe trial. They were told the purpose of this trial was to test their "intuition" by having them go to where they expected the invisible platform to be on the next trial. In this trial, the participant began at the center of the maze, facing east. Every arm had a sphere at its end, 5 of which were white and 1 of which was dark grey. This trial was designed to determine if the participants associated the dark gray sphere with the hidden platform or were biased by other aspects of the maze environment. The participants were asked to pick up the controller and go to the arm where they anticipated the platform would be hidden. Upon reaching the end of the arm, an audible beep was played, and movement was no longer possible.

In order to confirm the participant's understanding of the task, the computer screen was turned off and three questions were asked: 1) whether the platform would always be hidden in

the same place. Participants had to answer "*yes"* to progress. 2) what to do on Find-it trials. Participants had to respond, "*locate hidden platform*". 3) what to do on Show-me trials. Participants had to respond, "*return to arm where the platform was previously located*". In cases where participants did not correctly answer these questions. The experimenter advised them of the correct answer following a script and confirmed the participants understanding before proceeding.

Testing. We assessed maze acquisition speed (latency) and strategy bias. The testing phase consisted of ten trials, divided into two blocks of five trials each. Each block had a different maze configuration. Each learning trial (referred to as a "Find-it" trial) was followed by an explicit probe trial referred to as a "Show-me" trial. The participants were informed that they would now alternate between ten Find-it and Show-me trials across two blocks. During "Find it" trials, participants were instructed to locate an invisible platform hidden at the end of a maze arm as fast and as efficiently as possible. When participants reached the end of the correct arm, the visible platform appeared below them, a same beep as in training was played, and a translucent barrier locked them in place. It was suggested that participants look around the environment from this location and inform the experimenter when they were ready to proceed. The experimenter reminded participants that unless otherwise stated, once they located the hidden platform, it would remain there in all subsequent trials. Furthermore, participants would always be informed whether they were entering a Find-it or Show-me trial. Each Find-it trial was followed by a Show-me trial that had no hidden platform. In the "Show-me" trials, participants were instructed to return to the location in the maze where they found the platform in the previous Find-it trial. An audible beep was played whenever the participant reached the end of their chosen arm, and a translucent barrier locked them in place.

During the first block of trials, "Find-it" trials were conducted with the participant at the end of the east arm, and the platform was hidden in the southwest arm (two arms to the left of the starting arm and marked with a dark gray sphere). For the "Show-me" trials, the participant

began at the end of the northwest arm, while the dark gray sphere was moved to the end of the west arm. By rearranging the maze and the starting position, it was possible to distinguish navigation strategies based on the arm chosen during the "Show-me" trial. Participants who maneuvered to the southwest arm were scored as using an allocentric-place strategy, as this was the original spatial location of the platform (but no longer the location of the dark gray sphere, and no longer two arms to the left). Participants who maneuvered to the west arm (now marked with a dark gray sphere) were scored as using an egocentric-cue strategy. Participants who maneuvered to the southeast arm making the same body-turn (two arms) were scored as using an egocentric-response strategy. Participants who returned to the original start arm or entered the two remaining arms were deemed to not have used a strategy.

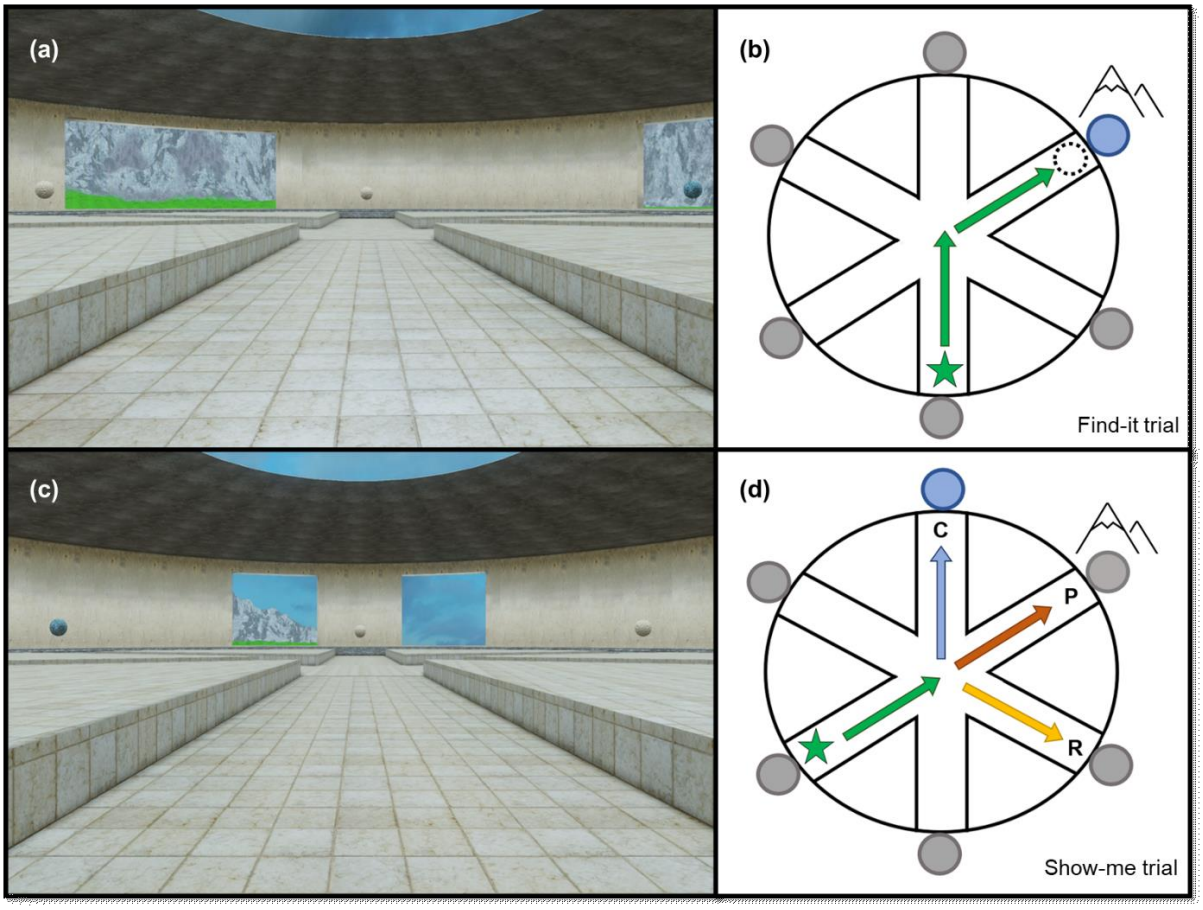

Figure 2. Images and illustrations of the Hex maze during the find-it and show-me trials in block 2. (a) View from the participants' perspective during the find-it trials. (b) Illustration of how participants must navigate to locate the hidden platform on Find-it trials. Find-it trials are conducted with a colored sphere

located on the same arm as the hidden platform, an extra-maze environment (e.g., mountains) in the background, and the participant starting two arms from the target arm. (c) View from the participants' perspective on show-me trials. (d) Illustration that shows how a participant's strategy is differentiated during a show-me trial. The participants started one arm to the left of where they started during the find-it trial. The colored sphere is placed one arm to the left of where it was placed during the find-it trial. The participant is using an egocentric cued strategy (C) if they navigate towards the colored sphere. The participant employs an allocentric-place (P) strategy if they navigate to the same spatial location (e.g., towards the mountain). If the participant navigates two arms away from their starting location, they are using an egocentric-response (R) strategy.

In order to reduce the potential effects of overtraining, two unique maze configurations were used. In advance of the sixth "Find-it" trial, participants were informed that the maze would be reconfigured and that the hidden platform would be moved. In the second block of paired trials, participants began the "Find-it" trial in the southwest arm, and the platform was hidden in the east arm. A blue sphere marked the east arm, which was two arms to the right of the start arm. In block 2, "Show-me" trials began at the west arm end and the blue sphere was relocated to the northeast arm end. The strategies used by participants were determined in the same way as during block 1 (Fig 2d).

In learning or "Find-it" trials, all three types of navigation strategies were convergent, but on probe or "Show-me" trials, they were divergent, showing the participants' choices of strategy. This divergence was not disclosed to participants in the "Show-me" trials. Two measures were generated; 1) strategy used in Show-me trials, and 2) latency in finding the hidden platform in Find-it trials.

Saliva collection and hormone quantification. The participants provided 2.5mL of saliva collected through passive-drooling using Salimetrics(C) SalivaBio's collection aid (part # 5016.04) into two sterile 1.5mL Eppendorf tubes. Within 30 minutes the samples were stored at -20C until they were analyzed. For saliva collection, participants were asked not to consume any drinks before or during the study and were provided with wooden stir sticks to chew on in order to stimulate saliva production.

Saliva samples were processed by the University of Montreal Regional Center for Mass Spectrometry and hormone levels were quantified. According to the center’s report, two different

LC-MRM assays were used: one for female saliva samples and the second one for male samples. Calibration curve standards and QC samples were prepared by serial dilution with decreasing concentration of T (females, males), P (females) and E2 (females) in artificial saliva. Calibration curve concentrations range varied according to each assay. Artificial saliva consisted of 0.1% bovine serum albumin in phosphate buffered saline at pH 7. All samples and standard were spiked with internal standards (β-estradiol d4 and Progesterone d9) at 150 pg/mL. Samples were defrosted and centrifuged at 16 000g, and 350uL of supernatant was applied on an SLE plate (Chem Elut S 400uL, Agilent) and absorbed using vacuum. The plate was incubated for 15min and eluted with 2 x 500uL of methyl tert-butyl ether by gravity. Eluents were dried under nitrogen stream, and resolubilized in 100uL of 50/50 MeOH/Water. Calibration curve concentration range was 1.17-150pg/mL for T in female samples, and 3.125-400pg/mL in male samples. In female samples, the calibration curve concentration range was 0.78-100pg/mL for E2 and 1.95-250pg/mL for P.

Reproducibility of the study was calculated using the QC samples and the overall CV was below 15%. Samples were analyzed over 4 days; the first 3 days were used for female samples and the fourth day was used for male samples. In female samples, reproducibility for each compound was E2 = 10.4%CV, P = 5.3%CV, and T = 9.1%CV. In male samples, the reproducibility for T was 0.9%CV.

Statistical Analysis. All statistical analyses were carried out using IBM SPSS Statistics (*Version 26*). To compare groups on demographic and psychometric data (*viz*., age, GPA, fluid IQ, perceptual speed, body mass index, and weekly hours of video game play) eight independent one-way analysis-of-variance (ANOVA) tests were performed. Two independent samples t-tests were performed to compare salivary levels of E2 and P between the NC and OC groups. In addition, a one-way ANOVA was used to examine differences in salivary P levels between the four NC and OC subgroups. Two additional one-way ANOVAs were used to

compare salivary T levels between groups (NC, OC, M) and subgroups (NC_{low}, NC_{high}, 2^{nd}Gen, 3^{rd}Gen, and M).

Because strategy use is categorical and thus non-parametric, chi-square analysis was used to determine if there were differences in the strategies used for each group and subgroup. Three 3x4 chi-squares were conducted for group, one for overall strategy (across all ten trials), one for block 1, and another for block 2. An additional three 5x4 chi-square tests were used to analyze if there was an association between subgroups (NC_{low}, NC_{high}, 2^{nd}Gen, 3^{rd}Gen, and M) and navigation strategies (allocentric, egocentric-response, egocentric-cue, and mixed).

In order to analyze maze acquisition, a two-factor repeated measures ANOVA with trial and group as factors and latency as the dependent variable was conducted. We hypothesized that by block 2 latencies would decrease given that participants would have learned how the task works and how to navigate using the controller during block 1. To analyze this, three paired sample t-tests (one for each the NC, OC, and M group) were performed to compare latencies from block 1 and block 2. Three one-way ANOVAs were performed to compare groups on overall mean latency and mean latency for block 1 and block 2. Similarly, three additional one-way ANOVAs were performed to compare subgroups on overall mean latency and mean latency for block 1 and block 2. Because equality of variance was violated a one-way ANOVA was inappropriate, and we therefore used a Brown-Forsythe test when analysing all latency data. When appropriate *post-hoc* comparisons were conducted using Tukey's HSD. In addition, we used partial eta squared (η_p^2) as a measure of effect size.

During different phases of the MC women have varying levels of E2 and P. Previous studies have shown that women perform differently on spatial tasks according to their MC phase (Hampson and Morley, 2013a; Hussain et al., 2016b). We therefore expected that results may differ between the NC subgroups. A planned comparison was conducted to contrast the NC_{low} and NC_{high} subgroups on latency results. Moreover, 2^{nd} and 3^{rd} generation OC have different

doses of EE and contain different progestins which vary in their affinity for AR (Beltz et al., 2015; Kuhl, 2005). Past studies have demonstrated that performance on spatial tasks can vary depending on the OC generation (Griksiene et al., 2018; Gurvich et al., 2020; Wharton et al., 2008). Thus, a planned comparison was conducted to analyze differences between the 2ndGen and 3rdGen subgroups.

Previous studies have demonstrated a correlation between performance on spatial tasks and endogenous hormone levels (Hampson, 2018; Pletzer et al., 2019b; Scheuringer and Pletzer, 2017). Thus, we examined whether salivary levels of E2, P, and T correlated with the strategies used during navigation or with latency to complete the navigation task using Pearson correlation coefficients. Only a subset of participants whose hormone levels were detected were included in this analysis. Where possible, we examined correlations between hormone level collapsed across all three groups (NC, OC, M), and then analyzed the same correlations within each group.

Results

Demographics and psychometrics. Table 1 presents the means and standard errors for each group, as well as the *p* values. Men spent significantly more time playing video games each week than women in the NC and OC groups (F (2, 164) = 16.80, p<0.01). Men played significantly more video games than NC women (p<0.01, 95% C.I. = -5.97, -2.03) and women taking OC (p<0.01, 95% C.I. = -6.52, -2.43). There were no statistically significant differences between groups on GPA (F (2, 140) = 1.89, p = 0.155), age (F (2, 163) = 1.79, p = 0.169), or BMI (F (2, 161) = 0.804, p = 0.449). We also found no statistically significant differences between groups on psychometric tests, namely Raven's matrices (F (2, 161) = 1.345, p = 0.263), the number of correct (F (2, 161) = 0.207, p = 0.813) or incorrect responses (F (2, 161) = 0.037, p = .964) on the IPT, or the number of responses on the IPT (F (2, 161) = 0.058, p = 0.943).

Table 1

Basic demographics and psychometric tests by group.

	Naturally-Cycling (NC)	Oral Contraceptives (OC)	Men (M)	*p*
Age (years)	22.07±0.40	21.43±0.28	22.45±0.41	0.17
GPA (4.3)	3.4±0.07	3.3±0.09	3.2±0.08	0.15
Body Mass Index (BMI)	22.99±0.56	22.7±0.61	23.68±0.52	0.45
Fluid IQ (Ravens)	35.61±3.01	37.13±2.81	43.30±4.51	0.26
Identical pictures task (Correct responses)	35.8±0.65	36.3±0.79	35.5±0.63	0.81
Identical pictures task (Incorrect responses)	2.40±0.35	2.53±0.54	2.43±0.35	0.96
Identical pictures task (# completed)	38.28±0.64	37.98±0.99	37.98±0.438	0.94
Weekly video game play (hours)	0.7h±0.20	0.2h±0.15	4.9h±0.98	**<0.01***

Note: M±SEM

Hormone analysis. For males, T was detected and quantified in all samples. For females, E2 was below the limit of quantification (LOQ) for most samples, while T and P was quantified in about half of the samples. Table 2 displays mean hormone levels for each group and each subgroup. No statistically significant differences were found between the NC and OC group for mean salivary E2 levels (t (21) = 0.265, p = 0.447). That said, salivary E2 levels were undetectable for the majority of samples (n=86). The mean salivary E2 values represent a small number of participants. Since E2 was detected in only three participants in the OC group, we did not analyze differences in OC subgroups for salivary E2.

The lower limit of quantification of E2 was 1.56pg/mL and for P was 0.98pg/mL. It was expected that women taking OC and NC women in the follicular phase would have levels that fall near or below these limits. As expected, E2 was not quantified in 94% of women taking OC, and P was not quantified in 67%. This is consistent with literature suggesting that women taking OC have levels similar to or lower than those observed in the follicular phase of NC women (Hampson, 2023). A large number of NC women from our sample also had levels of E2 and P that fell below the limits of quantification. This would be expected if these women were in the

follicular phase (Hampson, 2020). These values confirm that women taking OC had low levels of endogenous hormones and that many NC women were in likely the follicular phase at the time of testing.

There was a statistically significant difference in salivary P levels between the NC and OC groups such that levels were higher in the NC than in the OC group (t (49) = 2.212, p = 0.032). In analyzing subgroups, there was a statistically significant difference in salivary P levels among subgroups (F (3, 47) = 33.77, p<0.01). Women in the NC_{high} subgroup had higher levels of salivary P compared to those in either the NC_{low} group (p<0.01, 95% C.I. = -49.63, -27.53), the 2ndGen subgroup (p<0.01, 95% C.I. = -45.50, -20.04), or the 3rdGen subgroup (p = 0.01, 95% C.I. = -50.79, -21.85). Men's salivary P levels were not quantified.

The lower limit of quantification for T was 1.17pg/mL. We expected, based on previous literature (Snihur and Hampson, 2012; Zimmerman et al., 2014), that salivary T would be lower in women taking OC. Average salivary T levels were slightly lower in women taking OC than in NC women, but this difference was not statistically significant. However, T was not quantified for 3 NC women, while T was not quantified in 16 women taking OC. These results suggest that in many women taking OC, salivary T fell below 1.17pg/mL. The average value for salivary T in women taking OC (2.38±2.89pg/mL) was based only on the portion of the sample for which T was actually quantified.

There was a statistically significant difference in salivary T levels among groups (F (2,142) = 365.23, p<0.01). Men had significantly higher levels of salivary T than both NC women (p<0.01, 95% C.I. = -48.90, -40.18) and women taking OC (p<0.01, 95% C.I. = -50.13, -40.29). There was also a statistically significant difference between subgroups for salivary T (F (4, 137) = 174.21, p<0.01). Men had significantly higher salivary T compared to the NC_{low} (p = <0.01, 95% C.I. = -50.10, -38.81) and NC_{high} (p<0.01, 95% C.I. = -52.89, -36.63) subgroups, and men also had higher salivary T levels compared to the 2ndGen (p<0.01, 95% C.I. = -52.19, -37.99) and 3rdGen subgroups (p<0.01, 95% C.I. = -53.33, -37.50).

Table 2

Summary of salivary E2, P, and T levels as measured by LC/MS.

	17β-estradiol (E2) (*pg/mL*)	Progesterone (P) (*pg/mL*)	Testosterone (T) (*pg/mL*)
	Naturally cycling women (NCW)		
NC_{low}	M/SD: **1.21±1.50** Range: 0.03-5.78 (*n*=15); Not detected: 27	M/SD: **3.04±1.94** Range: 0.17-7.17 (*n*=18); Not detected: 24	M/SD: **3.13±2.42** Range: 0.10-11.62 (*n*=40); Not detected: 2
NC_{high}	M/SD: **1.64±1.11** Range: 0.81-3.25 (*n*=5); Not detected: 10	M/SD: **41.62±19.25** Range: 12.34-70.77 (*n*=15); Not detected: 0	M/SD: **2.84±1.85** Range: 0.27-7.52 (*n*=14); Not detected: 1
Group total:	M/SD: **1.32±1.39** Range: 0.03-5.78 (*n*=20); Not detected: 37	M/SD: **20.57±23.34** Range: 0.17-70.77 (*n*=33); Not detected: 24	M/SD: **3.06±2.23** Range: 0.102-11.62 (*n*=54); Not detected: 3
	Women taking oral contraceptives (OC)		
2nd gen	*NA*	M/SD: **8.66±3.34** Range: 0.036-37.26 (*n*=11); Not detected: 21	M/SD: **2.51±3.74** Range: 0.16-17.28 (*n*=20); Not detected: 12
3rd gen	*NA*	Mean/SD: **5.30±4.72** Range: 0.21-13.85 (*n*=7); Not detected: 13	M/SD: **2.24±1.35** Range: 0.31-4.55 (*n*=16); Not detected: 4
Group total:	M/SD: **1.10±0.71** Range: 0.46-1.87 (*n*=3); Not detected: 49	Mean/SD: **7.35±9.11** Range: 0.036-37.26 (*n*=18); Not detected: 34	M/SD: **2.38±2.89** Range: 0.16-17.28 (*n*=36); Not detected: 16
	Men (M)		
Group total:	*NA*	*NA*	M/SD: **47.60±15.54** Range:15.65-87.35 (*n*=53); Not detected: 0

Hex Maze.

Strategy use. There were no statistically significant differences in the distribution of strategies used among groups for all ten trials (χ^2 (6, *n*=161) = 3.73, *p*= 0.713), in block 1 (χ^2 (6, *n*=161) = 9.39, *p*= 0.153) or in block 2 (χ^2 (6, *n*=161) = 2.21, *p*= 0.899; Fig 3. We also found no statistically significant differences in the distribution of strategies used among subgroups for all ten trials (χ^2 (12, *n*=161) = 6.160, *p*= 0.908), in block 1 (χ^2 (12, *n*=161) = 11.359, *p*= 0.498) or in block 2 (χ^2 (12, *n*=161) = 7.120, *p*= 0.850).

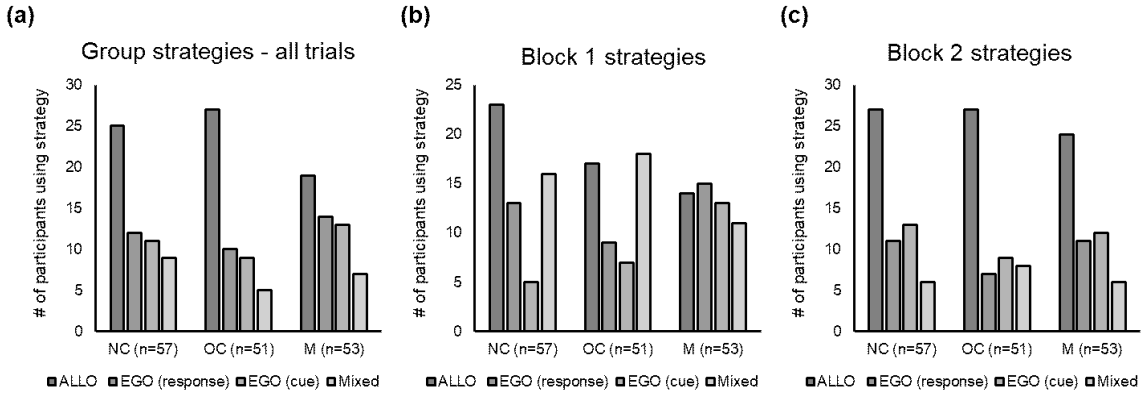

Figure 3. Bar graphs showing the distribution of strategies used by each group (a) across all ten trials, (b) in block 1, and (c) in block 2. ALLO represents an allocentric strategy, EGO (response) represents an egocentric-response strategy, EGO (cue) represents an egocentric-cued strategy and mixed represents participants who used more than a single strategy during navigation.

Latency data loss. A computer-related issue caused a loss of latency data for 22 participants who participated in the earlier stages of the study. Consequently, the final sample size per group (NC = 47, OC = 42, M = 50) and subgroup (NC_{low} = 34, NC_{high} = 13, 2^{nd}Gen = 27, 3^{rd}Gen = 15, M = 50) for the latency data analysis was reduced. The strategy use variable was manually recorded rather than by our testing software, therefore no data was lost on that measure.

Maze Acquisition. There was a statistically significant interaction between the effects of group and trial (F (18, 1224) = 1.78, p = 0.02). Men had shorter latencies than NC women on trials 1 (p<0.01), 2 (p=0.04), 3 (p=0.02), 4 (p=0.02), 5 (p<0.01), and 6 (p<0.01). Men also had shorter latencies than women taking OC on trials 2 (p=0.02), 5 (p=0.02), and 6 (p=0.047). Women taking OC had shorter latencies than NC women on trial 4 (p=0.03). There was also a main effect of group *(*p<0.01) such that the NC group had longer latencies than other groups. There was a main effect of trial *(*p<0.01) such that latencies decreased from trial 1 to trial 10 (Fig 4a). In addition, latency from block 1 to block 2 was reduced for all three groups (NC; t (46) = 8.624, p=<0.01; OC; t (42) = 8.598, p<0.01; and M; t (49) = 7.469, p=<0.01; Fig 4b).

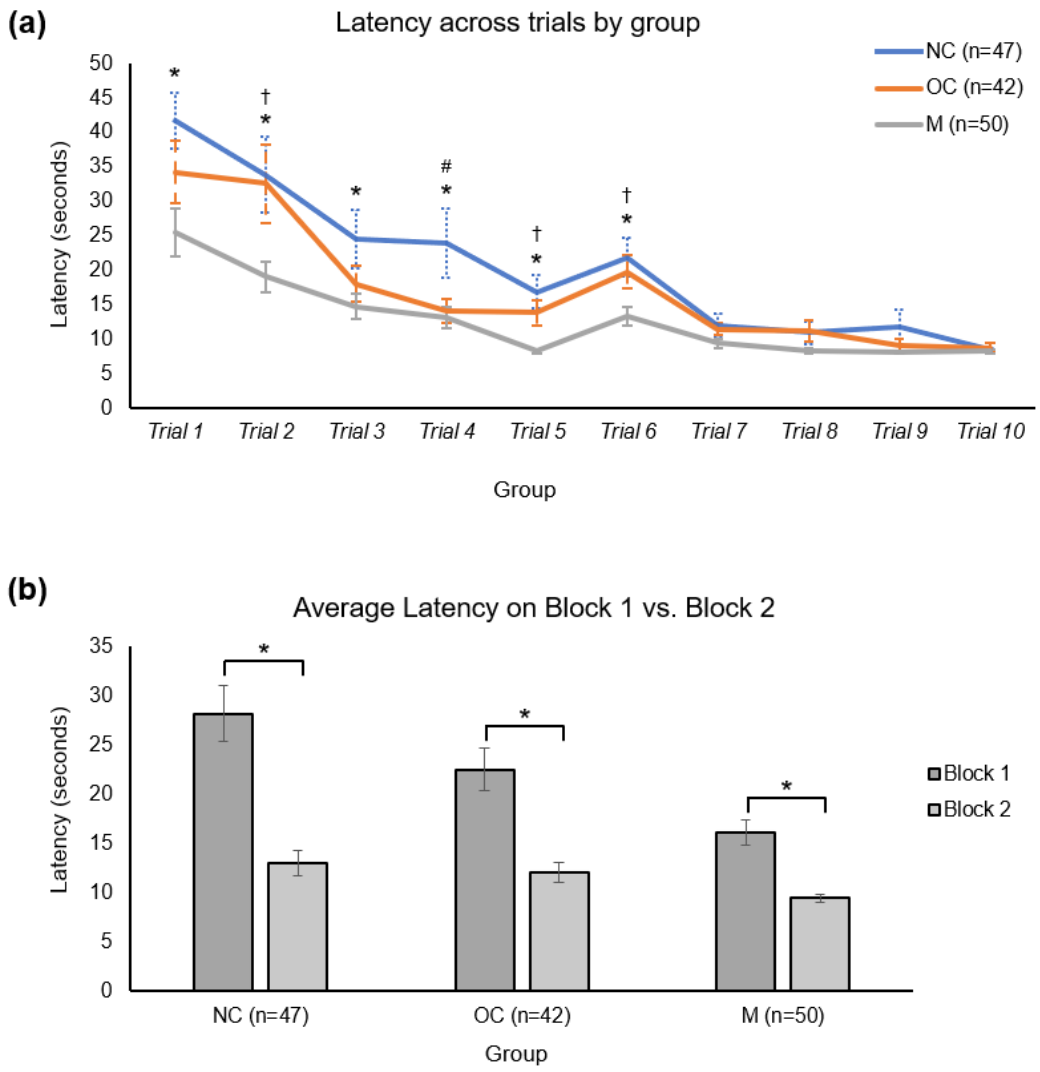

Figure 4. (a) A line graph representing the change in latency from trial 1 to trial 10 for each group: naturally-cycling women (NC), women taking oral contraceptives (OC) and men (M). Statistically significant differences between M and NC groups are marked with (*). Statistically significant differences between the M and OC groups are marked with (†). Statistically significant differences between the NC and OC groups are marked with (#). (b) A bar graph showing the difference between latency (measured in seconds) on block 1 compared to latency on block 2 for each group (NC / OC / M). Error bars represent standard error of the mean.

Latency. There was a statistically significant difference between groups on overall latency (F (2, 96.41) = 8.316, p<0.01, η_p^2 = 0.11), block 1 latency (F (2, 100.62) = 8.094, p<0.01, η_p^2 = 0.11), and block 2 latency (F (2, 91.99) = 3.912, p = 0.023, η_p^2 = 0.06). Compared to NC women, men had significantly shorter overall latencies (p = <0.01, 95% C.I. = 3.27,12.29), block 1 latencies (p = <0.01, 95% C.I. = 4.99,19.12), and block 2 latencies (p = 0.020, 95% C.I. =

0.46, 6.55). There were no statistically significant differences between women taking OC and men on overall latency, or latency during block 1 and 2. There was a statistically significant effect of subgroup on mean latency *(F* (4, 69.55) = 4.87, *p*<0.01, $\eta_p^2 = 0.13$). Men had shorter latencies than women in the NC_{low} subgroup (*p*=<0.01, C.I. = 2.42, 13.78) as well as the 2ndGen subgroup (*p*=0.04, C.I. = 0.241, 12.55). Planned comparisons revealed that there was no statistically significant difference between the NC_{low} and NC_{high} groups (*t* (23.54) = 0.318, *p*= 0.754). However, there was a statistically significant difference between the 2ndGen and 3rdGen groups (*t* (39.82) = 2.28, *p*=0.03) such that the 2ndGen group had longer overall mean latencies (M = 19.17, SD = 9.93) compared to the 3rdGen group (M = 13.89, SD = 5.05).

Block-wise latency. In block 1, there was statistically significant effect of subgroup on mean latency (*F* (4, 56.70) = 4.01, *p* = <0.01, $\eta_p^2 = 0.12$). Men had significantly shorter latencies than the NC_{low} subgroup (*p*=<0.01, C.I. = 3.33, 21.22). Planned comparisons revealed that there was no statistically significant difference between the NC_{low} and NC_{high} groups (*t* (18.74) = 0.125, *p*= 0.902) nor between the 2ndGen and 3rdGen groups (*t* (39.23) = 1.70, *p*=0.097). In block 2, there was statistically significant effect of subgroup on mean latency (*F* (4, 75.342) = 3.262, *p* = 0.01, $\eta_p^2 = 0.09$). Again, men had significantly shorter latencies than the NC_{low} subgroup (*p*=0.04, C.I. = 0.10, 7.74). Planned comparisons revealed that there was no statistically significant difference between the NC_{low} and NC_{high} groups (*t* (29.65) = 0.680, *p*= 0.502). However, the 2ndGen group had longer block 2 latencies (M = 13.49, SD = 7.39) compared to the 3rdGen group (M = 9.46, SD = 2.27; *t* (33.79) = 2.624, *p*=0.01).

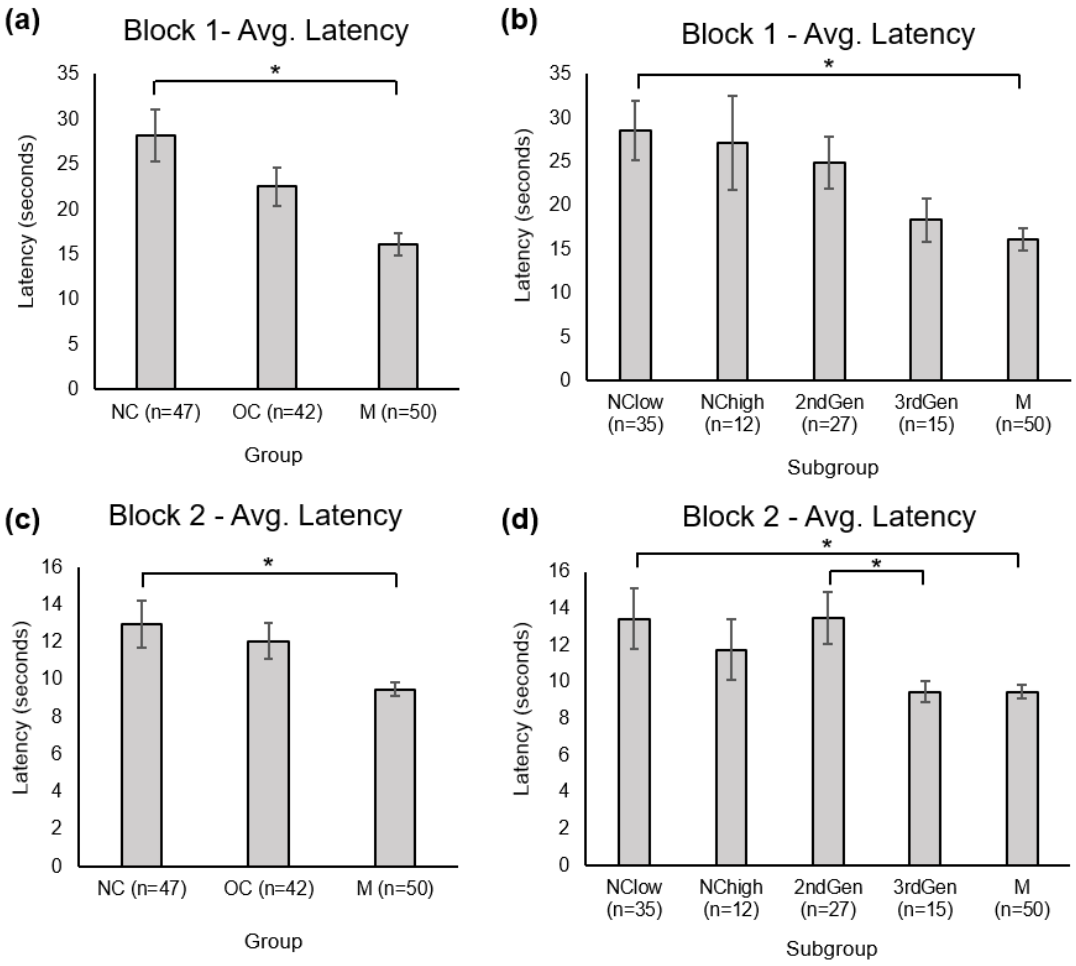

Figure 5. Bar graphs representing average latency to complete the Hex maze for NC, OC, and M groups during (a) block 1 and (c) block 2. Subgroups (NC_{low}, NC_{high}, 2^{nd}Gen, 3^{rd}Gen, and M) latency is shown for (b) block 1 and (d) block 2. Error bars represent standard error of the mean.

Maze performance and salivary hormone levels. There were no statistically significant correlations between salivary E2 levels and latency (total, or block-wise) nor with strategies used within the Hex maze (Table 3). When salivary P levels were collapsed across all women, there was a statistically significant negative relationship with salivary P levels and the use of an allocentric strategy (r (65) = -0.32, p = 0.01; Fig 6a). We also found a positive correlation between salivary P and the use of an egocentric-response strategy r (65) = 0.26, p = 0.04 (Fig 6b). Lastly, there was a statistically significant positive correlation between salivary P collected from those in the NC group and the use of an egocentric-response strategy (r (33) = 0.36, p = 0.04; Fig 6c). Collapsed across all women and within both the NC and OC groups,

there were no statistically significant correlations between salivary P levels and latency (total, or block-wise).

There were no statistically significant correlations between salivary T levels and strategies used within the Hex maze. When salivary T levels were collapsed across all groups, we observed a statistically significant correlation between salivary T and latency (Fig 6d). Salivary T was negatively correlated with overall latency (r (124) = -0.29, p<0.01), block 1 latency (r (124) = -0.28, p<0.01), and block 2 latency (r (124) = -0.25, p<0.01). However, when the NC, OC, and M groups were analyzed individually, there were no statistically significant correlations between salivary T and latencies (total or block-wise).

Table 3

Pearson correlations (*r*) between salivary hormone levels and Hex maze performance.

	Strategies used				*Latency to find hidden platform*			
	Allo-Place %	**Ego-Response %**	**Ego-Cue %**	***n***	**Overall**	**Block 1**	**Block 2**	***n***
				Entire Sample				
***T*†**	-0.143	0.065	0.106	142	**-0.294****	**-0.268****	**-0.253****	124
				Naturally-cycling women (NC)				
P	-0.340	**0.357***	0.083	33	0.015	0.043	-0.070	28
T	-0.128	0.040	0.170	54	-0.023	-0.019	-0.025	44
				Women taking oral contraceptives (OC)				
P	0.527×	-0.382×	-0.312×	12	-0.223	-0.251	-0.117	14
T	-0.141	0.129	0.048	35	-0.105	-0.057	-0.178	30
				All women				
E2	0.102	-0.274	0.298	28	-0.113	-0.159	-0.026	18
P	**-0.317***	**0.258***	0.154	65	0.006	0.050	-0.095	42
T	-0.133	0.074	0.120	89	-0.034	-0.008	-0.084	74
				Men (M)				
T	-0.052	0.063	0.020	53	-0.165	-0.113	-0.256	50

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
† = Only salivary T levels were measured in the entire sample.
× = small sample size

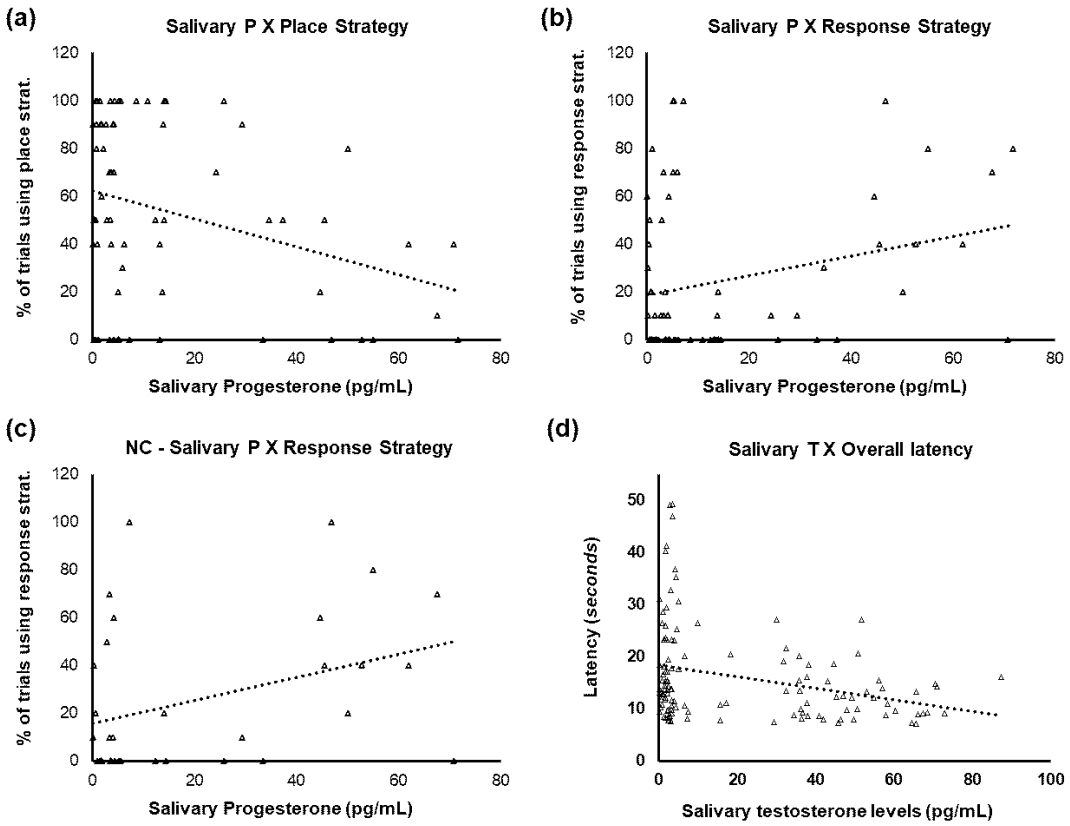

Figure 6. Scatterplot of latency (in seconds) results by endogenous salivary hormone levels (pg/mL). The linear trendlines represent the simple correlation. (a) salivary P levels collapsed across all women and its negative relationship with the use of % of trials out of ten where an allocentric strategy was used. (b) salivary P levels collapsed across all women and its positive relationship with the use of % of trials out of ten where an egocentric-response strategy was used. (c) salivary P levels in naturally-cycling women (NC) only, and its positive relationship with the use of % of trials out of ten where an egocentric-response strategy was used. (d) salivary T levels collapsed across all participants and its negative relationship with overall average latency to complete the Hex maze over ten trials.

Discussion

This study examined the role of biological sex as well as both endogenous and exogenous hormones on spatial navigation within a virtual Hex maze task. It was anticipated that men would complete the maze with shorter latencies than women (both NC and taking OC). There was a statistically significant difference between groups for latency to complete the maze. As was anticipated, men had shorter latencies than NC women. This was observed across all 10 trials (total latency) as well as within each testing block independently. Counter to expectations, these differences were not observed between men and women taking OC.

The fact that men had shorter latencies than NC women is consistent with several other studies which have demonstrated shorter latencies for men compared to women on navigation tasks (Daugherty et al., 2015; Gazova et al., 2013; Korthauer et al., 2017; Mueller et al., 2016; Piber et al., 2018; van Gerven et al., 2012). A more nuanced effect on overall latency was observed in subgroups. Men had shorter latencies compared to NC_{low} and 2^{nd}Gen subgroups but not the NC_{high} or 3^{rd}Gen subgroups. When latency was examined for each block independently, men only had significantly shorter latencies than the NC_{low} subgroup. This shows that women's hormonal milieu is an important factor when examining sex differences in latency during virtual navigation.

Previous studies have shown that whether a sex dependent effect is observed can depend on a women's MC phase (Hampson, 1990a, 1990b; Hampson and Morley, 2013b). For example, a study by Hampson and Morley, (2013) found that men and women performed equally on a spatial working memory task when women were tested during MC phases when E2 was low. However, when tested during high-E2 phases, women had better performance compared to men. Thus, whether a difference between men and women is observed is sensitive to women's ovarian hormone levels. We observed a similar trend, insofar as a difference was found between men and the NC_{low} subgroup, but not NC_{high} subgroup who had elevated levels of P.

It was also hypothesized that latency would correlate with salivary levels of E2, P, and T. We therefore examined the relationship between latency and salivary levels of these gonadal hormones. There was no relationship between salivary E2 or P levels and overall latency or block-wise latency. It should be noted here that few women had detectable levels of salivary E2 thus this test lacked power to detect a difference. We amalgamated salivary T levels from our entire sample. There was a significant negative correlation with latency such that overall, block 1, and block 2 latencies decreased as salivary T increased. These findings are similar to Bernal

et al. (2020) who found a significant positive relationship between accuracy in a navigation task when salivary T levels were collapsed across men, women taking OC, and NC women. This relationship was no longer statistically significant when each group was considered separately as was the case in the current study.

Previous studies have demonstrated that in men, performance on navigation tasks is either improved by T (Choi and Silverman, 2002; Driscoll et al., 2005) or unaffected by T (Burkitt et al., 2007). In women, elevated T improves (Burkitt et al., 2007) or has no effect on navigation performance (Choi and Silverman, 2002; Driscoll et al., 2005; Scheuringer and Pletzer, 2017). Women with short CAG repeats, which increase AR sensitivity, perform better on virtual navigation tasks compared to women with longer CAG repeats, who are less sensitive to androgens (Nowak et al., 2014). This latter study suggests that women's performance on virtual navigation tasks can be partially influenced by androgens such as T. Thus, our findings agree with the studies above that have shown that, overall, increased T improves navigation performance, but this relationship will require further investigation.

Consistent with past studies (Quaiser-Pohl et al., 2006; Terlecki et al., 2011; Terlecki and Newcombe, 2005), we showed that college-aged men tend to play significantly more video games than college-aged women. It is important to note that previous studies have shown that video gaming experience does not mediate the sex difference that is observed between men and women on virtual navigation tasks (Harris et al., 2022, 2019; van Dun et al., 2021). Nevertheless, it cannot be discounted that this may factor into why men had shorter latencies in the task presented here.

There were no statistically significant differences between women taking OC and NC women on latency. This is consistent with Patel et al. (2022) who did not observe any differences between women taking OC and NC women on two different virtual navigation tasks. Patel et al. (2022) did note, however, that those taking OC (first generation; norethindrone acetate) performed more similarly to women with high salivary E2 than to those with low salivary

E2. We were not able to detect E2 in enough women to determine whether our results are consistent with this finding. Bianchini et al. (2018) showed that women taking OC (3rd generation; gestodene) learned an eight-step sequence path in fewer trials than NC women in the follicular or the luteal phase. Similar to Bianchini et al. (2018), in our study the 3rdGen subgroup did have shorter latencies than women in both NC subgroups, but these differences were not statistically significant. Gurvich et al. (2020) found a difference between women taking OC containing 2nd generation progestins (androgenic), compared to those taking OC containing 4th generation progestins (anti-androgenic). They found that those using 2nd generation progestins had better performance on a navigation-related task (e.g., finding and recalling the location of a hidden pathway). However, none of the studies mentioned in this paragraph contrasted 2nd and 3rd generation formulations directly, which vary in their androgenicity.

It was hypothesized that women taking 2ndGen OC would have shorter latencies compared to those taking 3rdGen OC due to their elevated androgenic activity. There was a statistically significant difference between 2ndGen and 3rdGen subgroups on overall and block 2 latency. Contrary to what was hypothesized, it was observed that those taking 3rdGen OC had shorter latencies than women taking 2ndGen OC. This observation is counter-intuitive given the correlation that was observed between salivary T and latency. That is, 3rdGen OC contain progestins that are less androgenic than those contained in 2ndGen formulations (Kuhl, 2005; Schindler, 2015). If T promotes a reduction in latency, the same would be expected of OC with greater androgenic activity (i.e., 2ndGen). Women administered exogenous T (Pintzka et al., 2016) and women who have CAG polymorphisms which increase AR sensitivity have improved navigation performance (Nowak et al., 2014). Thus, given the known role of T in promoting spatial navigation performance in women, it was anticipated that women taking 2ndGen OC formulations would have better performance. However, this hypothesis was not born out in the current study.

It is possible that there are elements other than progestin androgenicity that are contributing to the difference observed between women taking 2nd and 3rd generation OC. For example, studies examining the impact of OC on mental rotation have shown an important role for the estrogenic effects of OC and not progestin type (Beltz et al., 2022, 2015; Hampson et al., 2022). Thus, perhaps the difference we have observed between 2nd and 3rd generation OC is related to their estrogenic components. Furthermore, 2nd and 3rd generation progestins also bind with varying capacity to mineralocorticoid receptors (Sitruk-Ware, 2005). Mineralocorticoid receptor activation has also been shown to impact women's navigation performance in a virtual Morris water maze task (Piber et al., 2016).

It was expected that men would use an allocentric (place) strategy and NC women would use egocentric (response and cued) strategies during navigation. It was also hypothesized that women taking 2ndGen OC would primarily use an allocentric strategy compared to NC women and women taking 3rdgen OC. Counter to our expectations, we observed no differences among any groups or subgroups on navigation strategy. That is, there were no statistically significant differences between NC women, women taking OC, and men in the frequency of use of allocentric or egocentric strategies. It was observed that, in most cases, all groups predominantly used an allocentric strategy, with some participants in each group using egocentric-response or cued strategies. The relative distribution of strategies changed from block 1 to block 2, however, there were no statistically significant differences among groups in strategies used during each block independently. In block 1, several participants within all groups used a mixed strategy. In block 2, however, participants within all groups predominantly used an allocentric strategy, with some minor variation. The changes in strategy between blocks suggests that in block 1 participants were applying different cognitive strategies as they learned the new maze environment. Yet in block 2, they applied their true dominant strategy as very few participants continued to use a mix of strategies.

No sex difference in navigation strategies was identified in this study which is consistent with some previous studies (Andersen et al., 2012; Bohbot et al., 2012; Gazova et al., 2013; Pletzer et al., 2019b; Rodgers et al., 2012; Scheuringer and Pletzer, 2017; van Gerven et al., 2012). This finding runs counter to other reports indicating that men tend to primarily use an allocentric strategy, while women tend to use egocentric strategies during navigation (Andersen et al., 2012; Astur et al., 2004; Galea and Kimura, 1993; Saucier et al., 2002; Spriggs et al., 2018). Only one previous study has used the Hex maze and they reported a small sex difference with 56% of their male sample using allocentric strategies, and 56% of their female sample using egocentric strategies (Spriggs et al., 2018). That study defined a "dominant strategy" as the strategy that was used in >50% of trials. Here, we used a much more stringent criteria (>80% of trials) to define a dominant strategy. These differences in defining dominant strategy, in addition to our study having nearly twice the sample size may account the discrepancy in observations.

The findings of the current study also differ from one of our previous studies (Hussain et al., 2016). We observed that women with elevated P predominantly used an allocentric strategy compared to those in phases with low serum P levels. Here, women with elevated salivary P levels did not show any statistically significant differences in strategies used from those with low or undetectable levels. It is possible that this difference is due to how navigation strategy was assessed by Hussain et al. (2016) compared to in the current study. Hussain et al. (2016) used a virtual radial arm maze in which navigation strategy was inferred based on the number of errors committed when the extra-maze environment was removed from sight. A greater number of errors indicated dependence on the spatial environment, thus, participants with more errors were coded as using an egocentric strategy. The Hex maze assesses navigation strategy directly, which may account for why our observations differ from our previous observations.

Statistically significant correlations were observed between P levels and strategies used. Salivary P levels were positively correlated with use of an egocentric-response strategy and

negatively correlated with use of an allocentric strategy. These findings are consistent with Scheuringer and Pletzer (2017) who demonstrated that P was positively correlated with better performance on an egocentric navigation task, and negatively correlated with performance on an allocentric navigation task. This is also consistent with findings in rodents showing that when E2 and P are administered together, female rats predominantly use a response strategy (Korol and Pisani, 2015; Lacasse et al., 2022b). Despite the positive relationship between P and egocentric-response strategies, we did not observe a statistically significant bias for egocentric-response strategies in the NC_{high} group where P was elevated.

We observed no relationship between salivary T and navigation strategies in men or women. The fact that T did not promote a bias towards using an allocentric strategy in men runs counter to findings in studies of male rodents. In gonadectomized male rats, administration of T dose-dependently increases the use of place memory (Spritzer et al., 2013; Wagner et al., 2018; Zhang et al., 2020). However, the finding that salivary T levels has no relationship to which strategy is used by women is consistent with previous studies (Hussain et al., 2016b; Scheuringer and Pletzer, 2017).

It is possible that we observed no differences in strategies due to the design of the virtual environment. While the Hex maze was designed to test the distribution of three unique navigation strategies, the environment itself may inadvertently promote the use of allocentric navigation. From any given position within the Hex maze the participant can view a large proportion of the maze. In addition, regardless of whether a participant is using a cued or response strategy, the extra-maze environment is still visible through windows no matter which arm they select. For example, the colored sphere cue is also surrounded by windows which allow participants to see the extra-maze environment behind the cue. While the design of the maze attempts to correct for this by not placing the most salient landmarks behind the cue (*e.g.,* the mountain sides, the island), the extra-maze environment is still present there. This may

unintentionally promote a more global processing of the maze which could be why the majority of participants in the study used allocentric navigation.

Conclusion.

We have demonstrated that biological sex, gonadal hormones, and oral contraceptives each have an interactive effect on spatial navigation on the Hex maze task. We have shown that men complete the task with shorter latencies than NC women but not women taking OC. This difference is specific to when NC women have lower levels of P. Salivary levels of T were also found to be negatively correlated with latency to complete the maze. While we observed differences related to latency, no differences were observed in strategies used during navigation between any of the groups in our study. It was also found that salivary P positively correlated with the use of an egocentric-response strategy, and negatively correlated with the use of an allocentric-place strategy. Nevertheless, we have demonstrated that endogenous and exogenous hormones indeed play a role in spatial navigation and should be considered when examining the effect of biological sex on virtual spatial navigation.

General Discussion

This thesis examined the role of endogenous and exogenous gonadal hormones on spatial navigation in female rats and women. Exogenous and endogenous hormones were

hypothesized to have an impact on memory bias during spatial navigation in rats and in humans.

In the first chapter, we built on previous work which had demonstrated that the use place and response memory were sensitive to gonadal hormone fluctuations that occur during the female rat estrous cycle (Korol et al., 2004b). Female rats use response memory when E2 levels are low, but place memory when E2 levels are high (Almey et al., 2014; Hussain et al., 2016a; Korol and Kolo, 2002; Quinlan et al., 2013, 2008). A single study had examined P's role in place and response memory (Korol and Pisani, 2015). Female rats were shown to have a shift towards response memory when given high E2 in addition to P. In chapter 1, our results replicated this previous finding. Thus, P might promote the opposite effect to that of high E2. The opposing effects of P to those of E2 have been demonstrated more broadly in studies of dendritic spine density (DSD; Woolley and McEwen, 1993), as well as other assessments of HPC-mediated spatial memory (Bimonte-Nelson et al., 2004b, 2003; Chesler and Juraska, 2000; Harburger et al., 2007). We also showed a time-dependent of P effect as well. A shift from place to response memory was only observed when P was administered 1hr and 4hrs before testing. According to these results, P acts rapidly to influence memory bias.

We then wanted to test whether similar results would be observed with exogenous hormones. A model of hormonal contraception for female rats was developed in chapter 2. EE and LNG alone or in combination produced acyclic vaginal cytology in female rats that differed from that of naturally cycling females. The administration of these drugs also reduced LH levels significantly. Based on our review of the literature and these finding we proposed ways to best model HC in rats.

Chapter 3 describes the findings of our research on memory bias in female rats using our HC model. As with endogenous gonadal hormones, exogenous hormones like EE and LNG also influence memory bias. Here, we showed that EE and LNG both influence memory bias but

do so in a manner that is different if they are administered alone versus when they are combined.

Chapter 4 examined how biological sex, gonadal hormones, and oral contraceptives affect spatial navigation in humans. Navigation strategies were not different between men and women, regardless of hormonal milieu. There was, however, a relationship between salivary P levels and women's navigation strategies. Although we found no sex difference in navigation strategies, there was a sex difference in performance. The task was completed more quickly by men than naturally cycling women, particularly when E2 and P levels were low. The latency to complete the task was not different between men and women taking OC. Based on the findings of this chapter, there are differences between men and women in spatial navigation, at least in terms of latency. The extent to which this sex-dependent effect is observed, however, depends largely on women's endogenous and exogenous hormones.

Endogenous and exogenous hormones in spatial navigation.

This thesis emphasizes that gonadal hormones, both endogenous and exogenous, affect spatial navigation both in female rats and in humans. There is one commonality between the results presented in chapters 1 and 3, despite the fact that the hormones used in each experiment were different. In both experiments, there was a difference between how endogenous and exogenous hormones affected memory bias based on whether hormones were administered alone or in combination. In chapter 1, administration of high levels of E2 promoted a bias towards place memory. In combination with P, however, high E2 promoted response memory. That P acts differently when combined with E2 compared to when its given alone has been shown in several previous studies (Becker and Rudick, 1999; Chesler and Juraska, 2000; Fernández-Ruiz et al., 1989; Harburger et al., 2008; Orr et al., 2012). Chapter 3 also showed a bias toward place memory when LNG was administered alone. Yet when LNG

was combined with EE, it promoted response memory. It is important to consider how hormones interact to affect cognition in light of these data.

Several studies have shown that E2 can affect the use of place and response memory in female rats at low or high doses (Almey et al., 2014; Hussain et al., 2016a; Quinlan et al., 2013, 2008). The administration of E2 on its own, however, only reflects diestrus/metestrus and proestrus phases in female rats. In this regard, chapter 1 builds on previous studies by including P and reflecting a similar state to when female rats enter estrus. Female rats show a bias towards using response memory when are in estrus (Korol et al., 2004b). The response memory bias observed during estrus is likely due to the interaction of E2 and P rather than solely due to changes in E2 levels. Combinations of hormones reflecting all stages of the estrous cycle of rats should be considered in future studies.

The findings presented in chapters 1 and 4 shared another common thread. In both studies, P was associated with response memory or an egocentric-response strategy. The findings of our studies are consistent with previous studies, both in female rats and in humans, which showed that P promotes response memory (Korol and Pisani, 2015; Scheuringer and Pletzer, 2017). Therefore, chapters 1 and 4 of this thesis provide additional insight into the role of P in spatial navigation, specifically in terms of memory bias and the timing of Ps effects.

The exogenous hormones that were used in chapter 3, EE and LNG, are a combination that reflect second generation OC. In this regard, what was observed in chapter 3 did not translate to what was observed in women using second generation OC in chapter 4. Specifically, female rats administered EE+LNG primarily used response memory. However, women taking second generation OC showed a bias towards using the opposite, an allocentric strategy. What was observed in female rats was therefore different than what was observed in women taking the same combination of exogenous hormones. The tests used for rats and the one used for humans, albeit similar, are not identical. The data from this thesis suggests that

while exogenous gonadal hormones do indeed impact spatial navigation in both female rats and in women, their effects in humans are different from those observed in rats.

Potential Mechanisms.

In light of the findings presented throughout this thesis, the question of mechanism inevitably arises. What role might endogenous hormones like E2 and P, or exogenous hormones like EE and LNG play at the mechanistic level when it comes to spatial navigation? These putative mechanisms were not directly explored in this thesis. The extant literature, however, allows for speculation based on previous findings. It would be expected that endogenous and exogenous hormones would exert their influence on memory bias in the HPC and DS. These regions are rich in ER (Almey et al., 2016, 2015, 2012; Quigley et al., 2021), PR (Brinton et al., 2008), and AR (Menard and Harlan, 1993; Sarkey et al., 2008), hormonal changes therefore have the potential to influence them.

Woolley and McEwen (1993) found that E2 promotes dendritic spine growth, while P attenuates it. By stimulating the transcription of brain-derived neurotrophic factor (BDNF), E2 increases DSD (Luine and Frankfurt, 2020). Increased hippocampal DSD is linked to improved HPC-mediated spatial memory (Frankfurt and Luine, 2015; Luine and Frankfurt, 2013). P treatment decreases the E2-dependent increase in BDNF within the HPC (Aguirre et al., 2010; Baudry et al., 2013; Gibbs, 2000). Furthermore, P antagonists prevent female rats' natural decline in DSD between pro-estrus and estrus (Woolley and McEwen, 1993). These findings are in agreement with those presented in chapter 1, indicating that P has an opposite effect to that of E2.

Hippocampal DSD increases during proestrus when levels of E2 are elevated (Woolley et al., 1990). However, DSD is reduced as female rats transition into estrus and levels of P increase (Woolley et al., 1990). Korol et al. (2004) established that the proestrus phase was associated with a place memory bias, whereas the estrus phase was associated with a

response memory bias. Together, these findings suggest that hippocampal DSD may be associated with memory bias. As a result, altering DSD in the HPC may be one mechanism by which E2 and P influence memory bias (see Gomez-Perales and Brake (2022) for review).

Hippocampal volume fluctuates across the MC and is positively correlated with levels of P (Lisofsky et al., 2015; Taylor et al., 2020). However, this P-associated increase in hippocampal volume is not observed in women who take second generation OC that contain EE and LNG (Taylor et al., 2020). To date, no studies have examined the effects of EE and LNG on hippocampal DSD. However, the same doses of EE and LNG used in chapter 3 significantly reduced hippocampal BDNF protein and mRNA levels (Simone et al., 2015). Further, in human studies, OC has been shown to reduce serum BDNF levels in women (Pluchino et al., 2009). It is therefore possible that EE and LNG can impact memory bias through their effects on BDNF, which in turn can affect hippocampal DSD.

The findings in chapter 1 could also be attributed to P's metabolite, ALLO. ALLO binds to $GABA_A$ receptors (Bitran et al., 1995; Brot et al., 1997) which are abundant within the HPC (Isaacson et al., 1993; Palpagama et al., 2019; Ruiz et al., 2003) and play an important role in regulating HPC-mediated spatial memory (Andrews-Zwilling et al., 2012; Dashniani et al., 2020; Paulsen and Moser, 1998; Saffarpour et al., 2017). A high dose of ALLO (2mg/kg) administered to female rats impaired HPC-mediated spatial memory as measured by the MWM (Johansson et al., 2002). In a study by Matthews et al. (2002), it was found that acute administration of ALLO (17 and 20mg/kg) impaired performance on the spatial memory component of the MWM without affecting non-spatial performance measures. Chronic administration of ALLO (20mg/mL via osmotic pump) impairs spatial memory performance in female mice in the MWM and also reduces their hippocampal volume (Bengtsson et al., 2016). Accordingly, ALLO's action within the HPC could contribute to the observations of chapter 1.

P and ALLO levels in plasma and in the HPC are also significantly reduced in female rats treated with EE (20 or 30µg), or LNG (60 or 125 µg), or both (Porcu et al., 2012; Santoru et

al., 2014). The serum concentrations of ALLO are also reduced in women taking OC (Follesa et al., 2002; Paoletti et al., 2004; Rapkin et al., 2006). Thus, suppression of P and ALLO may have contributed to what we observed in chapter 3 in female rats and in chapter 4 in women taking OC.

Endogenous and exogenous hormones may also exert their effects through dopaminergic transmission in the striatum. Dopaminergic transmission in the DS is increased in response to systemic E2 treatment or direct infusions of E2 (Becker, 1990; Becker and Rudick, 1999; Shams et al., 2018, 2016). The administration of either a D1 receptor antagonist or a D2 receptor antagonist switched OVX female rats from a response memory bias to a place memory bias (Quinlan et al., 2008). Quinlan et al. (2013) reported similar results with direct infusions of a D1 receptor antagonist into the DS. Response memory is also impaired by direct infusions of E2 into the striatum (Zurkovsky et al., 2011). It would therefore appear that dopamine release in the DS is regulated by E2 and is involved in response memory. P treatment (500μg) increases striatal dopamine (DA) levels, but only when rats are primed with EB (5μg). When administered alone, P reduces striatal dopamine D2-family receptors. However, when E2 is given for 3 days prior to P treatment, striatal D2-family receptor numbers increase. These findings not only show that P affects striatal DA receptors, but that its action is dependent on E2 as was demonstrated in chapter 1.

In female rats, striatal DA levels are decreased by mestranol, a pro-drug for EE, and norethindrone, an androgenic progestin (Jori and Dolfini, 1976). Later, the same authors found that chronic exposure to these drugs increased tritiated-tyrosine conversion into tritiated-dopamine, indicating higher tyrosine utilization (Algeri et al., 1976). The total number of dopaminergic cells in the substantia nigra of female rats treated with LNG (30mg over 17 days) was also significantly reduced in comparison with naturally cycling female rats (Hilz et al., 2021). According to these findings, dopaminergic transmission is also influenced by EE, LNG as well as other androgenic progestins (NET).

Using Fluorine-18-methyltyrosine Positron Emission Tomography (18F-FMT PET), Taylor et al. (preprint) found that women taking OC had increased striatal dopamine synthesis capacity compared with NC women. The effect was most pronounced in the dorsal caudate, and it was associated with greater cognitive flexibility in a task-switching paradigm compared to NC women. These DA related findings offer yet another putative mechanism to be further explored related to OC and changes in memory bias during spatial navigation.

Conclusion.

It is clear from the data presented in this thesis that both endogenous and exogenous hormones play an important role in spatial navigation. In female rats, hormones like E2, P, EE, and LNG all impact memory bias during spatial navigation in their own way. Moreover, it was shown when these hormones are administered in combination their effects are different than when administered alone. In humans, there are sex differences in spatial navigation performance but not necessarily in navigation strategies. The sex differences observed in navigation performance also largely depend on women's hormonal state. For example, men were only different from naturally cycling with low E2 and P levels. Moreover, men did not differ from women taking OC which indicates the importance of considering not only endogenous but also exogenous hormones when studying sex differences in spatial navigation.

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