Neuromodulatory effects of 17β-estradiol on synaptic transmission in the entorhinal cortex

Ariel Andrius Batallán Burrowes

A Thesis
In the Department
Of
Psychology

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

December 2022

CONCORDIA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

By: Ariel Andrius Batallán Burrowes

Entitled: Neuromodulatory effects of 17β-estradiol on synaptic transmission in the entorhina

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

Doctor Of Philosophy (Psychology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair
Dr. David Walsh

_____ Thesis Supervisor
Dr. C. Andrew Chapman

_____ Thesis Supervisor
Dr.

_____ Thesis Supervisor
Dr.

_____ Examiner
Dr. Wayne Brake

_____ Examiner
Dr. David Mumby

_____ Examiner
Dr. Richard Courtemanche

_____ Examiner
Dr.

_____ External Examiner
Dr. Tak Pan Wong

Approved by

_____
Dr. C. Andrew Chapman , Graduate Program Director

«Date»

_____
Dr. , Dean

ABSTRACT

The neuromodulatory effects of 17β-estradiol on synaptic transmission in the entorhinal cortex

Ariel Andrius Batallán Burrowes, PhD
Concordia University, 2022

Changes in endogenous concentrations of estrogens can influence cognition in humans and animals. Estrogens are thought to affect cognition by modulating synaptic and neuronal function. The hippocampus is important for learning and memory, and estrogens can enhance synaptic transmission in that region. The entorhinal cortex is involved in object recognition, olfaction, and navigation, and provides the hippocampus with most of its cortical sensory and associational inputs. However, the contribution of estrogens to the modulation of entorhinal cortex function is unclear.

The work presented herein used electrophysiological and protein quantification techniques to assess the effects of estrogens in the entorhinal cortex. Electrically evoked postsynaptic field potential recordings (fEPSPs) were used in the first experiments to assess the rapid functional effects of acute 17β-estradiol (E2) application on excitatory glutamatergic transmission in the superficial layers of the entorhinal cortex *in vitro*. Results demonstrated that E2 reversibly facilitates fEPSPs via activation of the membrane bound G protein-coupled estrogen receptor-1 (GPER1). The second group of experiments further examined the cellular mechanisms of this GPER1-mediated facilitation using whole-cell patch-clamp recordings to measure changes in intracellular postsynaptic currents following E2 exposure. These recordings showed significant reductions in NMDA receptor-mediated excitatory postsynaptic currents and $GABA_A$ receptor-mediated inhibitory currents following E2 exposure. This suggests that E2 rapidly suppresses inhibition to facilitate excitatory synaptic transmission.

Diminished estrogenic function following natural or surgical menopause can induce cognitive alterations which may be ameliorated by estrogen supplementation. The loss of estrogens can alter cholinergic function throughout the brain and this may contribute to cognitive alterations. The last group of experiments explored in this thesis tested the effects of prolonged estrogen loss on cholinergic function in the entorhinal cortex. Western blot protein quantification

showed significant reductions in acetylcholinesterase and M_1 receptor protein following ovariectomy when compared to intact rats, and E2 supplementation following ovariectomy prevented these effects. Application of eserine, an acetylcholinesterase inhibitor, markedly reduced fEPSP amplitudes in all groups, but the reduction observed in ovariectomized rats was significantly reduced comparted to intact and E2 supplemented groups. Together, these works provide evidence for the modulation of synaptic function by 17β-estradiol in the entorhinal cortex.

Acknowledgements

The completion of this thesis has been long, challenging, and enlightening. It is the culmination of the blood, sweat, tears, and determination that I have invested in my career, education, and personal growth. However, to say that I completed this on my own, would be a disservice to the people that helped and supported me during these stressful and wonderful times.

The CSBN has been an enriching and supportive environment during my time at Concordia, and its members have contributed to my success. As my supervisor, Dr. Andrew Chapman has provided me with mentorship, tools, and opportunities to pursue research that is important to me. Without his confidence and support, I would not have achieved as much academically as I have. My lab mates Dr. Iulia Glovaci, Marcus Suvanto, and Dr. Olayemi Joseph Olajide, helped me acclimate, design, and conduct experiments, and aided me through my experimental struggles. Dr. Wayne Brake has also served as a mentor and supported my development as a researcher, providing insights and knowledge of endocrinology essential to the early stages of this work. The members of his lab, Dr. Waqqas Shams, Jesse Lacasse, Conall Mac Cionnaith, Eammon Gomez-Perales, and Collin Gagne, facilitated my development of skills and comprehension of literature on ovarian hormone function through countless discussions. I thank all of them for the help they've provided.

I would also like to thank all my friends from the CSBN, Dr. Mandy Lecocq, Dr. Alex Chisholm, Dr. Lindsay Sparks, Dr. Milan Valyear, Dr. Franz Villaruel, Christine Gerson, Maria Santaguida, Dr. Sara Hines, Dr. Kathleen Kennedy-Turner, Smita Patel, Lauren Buynack, Dr. Sara Ferraro, Dr. Nuria de Zavalia, Cassandra Goldfarb, Dilara Gostolupce, Dr. Megan Barlow, and any others that I may have forgotten that served as key social support. They were always willing to share and discuss the unique experiences and difficulties of graduate school.

A special thank you to Dr. Czarina Evangelista and our three cats, Nona, Rwby, and Didi who were there every day and provided emotional support, comfort, patience, and understanding when the stresses of research and academics were high. A gigantic thank you to my family, my parents, grandparents, brother, aunts, and uncles who have supported me emotionally and financially during this time, despite being in a different country. They have always been there for me and have given me the drive and determination to pursue my interests and goals, and catch me anytime I stumble or fall.

Without these people, I would not be who I am today. I am grateful to have had so many mentors, peers, and friends enrich my journey at Concordia, and I hope to keep them with me on the rest of life's journey.

Contribution of Authors

The work described within contains contributions from various authors, including myself and Dr. C. Andrew Chapman. All experiments were possible through funding from the Natural Sciences and Engineering Research Council (NSERC) obtained by Dr. Chapman, and the Center for Studies in Behavioural Neurobiology (CSBN).

Chapter 2: G protein-coupled estrogen receptor-1 enhances excitatory synaptic responses in the entorhinal cortex

- Ariel Andrius Batallán Burrowes was involved in the design of experiments, collected and analyzed the majority of data, and significantly contributed to the writing of the publication.
- Adithi Sundarakrishan aided in the collection of excitatory postsynaptic field potential recordings to assess the acute effects of estrogen receptor α and G protein-coupled estrogen receptor-1.
- Camille Bahour aided in the collection of excitatory postsynaptic field potential recordings to assess the acute effects of progesterone and allopregnanolone on excitatory synaptic transmission in the entorhinal cortex.
- Dr. C. Andrew Chapman contributed to the design of experiments, aided in the collection and analysis of data, and contributed significant portions of writing and revision for the final publication.

Chapter 3: 17β-estradiol reduces $GABA_A$ and NMDA receptor-mediated synaptic currents in the lateral entorhinal cortex

- Ariel Andrius Batallán Burrowes was involved in the design of experiments, recorded and analyzed the majority of electrophysiological data, and contributed to the writing of the chapter.
- Elyse Moisan aided in the collection of whole-cell patch-clamp recordings used to assess the effect of acute estrogen exposure on excitatory AMPA and NMDA and inhibitory $GABA_A$ postsynaptic currents in the entorhinal cortex. She also contributed to the analysis of data and initial drafting of the chapter.

- Dr. C. Andrew Chapman was involved in the design and preparation of novel materials used in the experiments. He also aided in data analysis, drafting, and revision of the chapter.

Chapter 4: Ovariectomy reduces cholinergic modulation of excitatory synaptic transmission in the rat entorhinal cortex

- Ariel Andrius Batallán Burrowes collected and analyzed a significant portion of the electrophysiological data included in the publication, was involved in the design of experiments, and contributed to the writing and revision of the publication.
- Dr. Olayemi Joseph Olajide assisted with the design of experiments, collected and processed tissue samples used for Western blots to quantify changes in cholinergic proteins in the entorhinal cortex. Furthermore, Dr. Olajide provided the initial written analysis and documentation of Western blot results included in the published chapter.
- Isabella Iasenza collected excitatory postsynaptic field potential recordings to assess the effects of ovariectomy on cholinergic transmission in the entorhinal cortex.
- Dr. Waqqas Shams conducted ovariectomies for the early stages of this study and provided training and guidance for me to conduct them independently.
- Francis Carter aided in the collection of excitatory postsynaptic field potential recordings for data included in the study of the effects of prolonged estrogen deprivation on synaptic function in the entorhinal cortex.
- Dr. C. Andrew Chapman was involved in the initial conceptualization of this project, contributed to the data collection and analysis, and contributed to the writing and revision of the publication.

Table of Contents

List of figures x

List of abbreviations xi

Chapter 1: *General Introduction* ***1***

Ovarian hormones and the reproductive cycle 3

Cyclic changes in cognition 5

Aging, reproductive senescence, and cognitive impairment 7

Estrogenic function in the brain 10

Estrogens and cholinergic function 12

The entorhinal cortex 14

Laminar and cellular organization 15

Estrogens in the entorhinal cortex 19

Experimental chapter summaries 21

Chapter 2: *G protein-coupled estrogen receptor-1 enhances excitatory synaptic responses in the entorhinal cortex* ***24***

INTRODUCTION 26

METHODS 28

Ovariectomy and estrogen implants 28

Slice preparation and recordings 29

Pharmacology 30

Data analysis 31

RESULTS 31

E2, progesterone, and allopregnanolone 31

Estrogen receptors 33

DISCUSSION 34

GPER1 signaling mechanisms 35

Progesterone 37

Functional significance 37

Chapter 3: *17β-estradiol decreases isolated GABA and NMDA currents in the lateral entorhinal cortex* ***47***

ABSTRACT 48

INTRODUCTION 49

METHODS 51

Ovariectomy and estrogen implants 51

Slice preparation 51

Electrophysiological recordings 52

Data analysis 53

RESULTS 54

AMPA receptor-mediated EPSCs 54

NMDA receptor-mediated EPSCs 55
GABA$_A$-mediated IPSCs 56

DISCUSSION 56
AMPA and NMDA receptor-mediated EPSCs 57
Modulation of GABA$_A$-mediated IPSCs 59
Functional implications 60

Chapter 4: *Ovariectomy reduces cholinergic modulation of excitatory synaptic transmission in the rat entorhinal cortex.* ***..... 68***

ABSTRACT 69

INTRODUCTION 70

METHODS 72
Subjects and surgery 72
Protein quantification 73
Electrophysiological recordings 75

RESULTS 76
Protein quantification 76
Effects of eserine on field EPSPs 77

DISCUSSION 78
Reductions in cholinergic synaptic proteins 79
Reduced effect of eserine on EPSPs 81

Chapter 5: *General discussion* ***..... 87***

Loss of estrogens and menopause 91

Impact of estrogens in the entorhinal cortex on cognitive function 92

Future Directions 93

References 96

List of figures

FIGURE 2.1. EFFECTS OF 17β- ESTRADIOL ON FIELD EPSPS IN THE ENTORHINAL CORTEX 40

FIGURE 2.2. EFFECTS OF PROGESTERONE AND ALLOPREGNANOLONE ON FIELD EPSPS 42

FIGURE 2.3. EFFECTS OF ER α (PPT) AND β (DPN) SPECIFIC ANTAGONISTS ON FIELD EPSPS. 44

FIGURE 2.4. EFFECTS OF SELECTIVE GPER1 ACTIVATION AND ANTAGONISM ON FIELD EPSPS. ... 46

FIGURE 3.1. REPRESENTATIVE TRACES OF MEMBRANE POTENTIAL RESPONSES FROM ENTORHINAL FAN AND PYRAMIDAL CELLS 63

FIGURE 3.2. EFFECTS OF 17β-ESTRADIOL ON AMPAR- AND NMDAR-MEDIATED EXCITATORY POSTSYNAPTIC CURRENTS 65

FIGURE 3.3. EFFECTS OF 17β-ESTRADIOL ON $GABA_AR$-MEDIATED INHIBITORY POSTSYNAPTIC CURRENTS 67

FIGURE 4.1. EFFECTS OF OVARIECTOMY AND 17β-ESTRADIOL SUPPLEMENTATION ON ACHE, CHAT, AND M_1 RECEPTOR PROTEIN CONCENTRATIONS 84

FIGURE 4.2. EFFECTS OF OVARIECTOMY AND 17β-ESTRADIOL SUPPLEMENTATION ON CHOLINERGIC MODULATION OF FIELD EPSPS 86

List of abbreviations

ACSF	artificial cerebrospinal fluid
CA1, CA3	*cornu Ammonis* 1, *cornu Ammonis* 3
cAMP	cyclic adenosine monophosphate
CB	calbindin
CNQX	cyanquixaline
DG	dentate gyrus
DMSO	dimethylsulfoxide
E2	estradiol, 17β-estradiol
EPSC	excitatory postsynaptic currents
fEPSP	excitatory postsynaptic field potentials
ER	estrogen receptor
ERK	extracellular-signal-regulated kinase
GABA	γ-Aminobutyric acid
GPER1	G protein-coupled estrogen receptor-1
HRT	hormone replacement therapy
I_h	hyperpolarization-induced current
IP_3	inositol triphosphate
IPSC	inhibitory postsynaptic current
LEC	lateral entorhinal cortex
LI-IV	layers 1-6
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEC	medial entorhinal cortex
NMDA	N-methyl-D-aspartate
PV	parvalbumin
SEM	standard error of the mean

OVX	Ovariectomy
WHIMS	Women's Health Initiative Memory Study

CHAPTER 1

GENERAL INTRODUCTION

Ovarian hormones are important for female maturation, reproduction, and the maintenance of physiological processes in humans and animals. Ovarian hormones fluctuate rhythmically and facilitate the regulation of the female reproductive cycle. Hormone fluctuations associated with the human menstrual cycle can influence attention, emotion regulation, spatial navigation, learning, and memory (Hamson et al., 2016; Newhouse & Dumas, 2015). Studies in animal models have reported similar findings, and link changes in ovarian hormone concentrations to changes in maternal care, pain sensitivity, spatial navigation, fear conditioning, object recognition, and social learning (Hara et al., 2015; Hussain et al., 2016). Cyclic variations in ovarian hormones also induce changes in neuroanatomy and synaptic function in various brain regions and are believed to underlie changes observed in cognition and behavior (Boyle et al., 2021; Engler-Chiurazzi et al., 2017). In the hippocampus, an area important for memory and spatial navigation, elevated levels of 17β-estradiol, a type of estrogen, can increase synaptic transmission and dendritic spine densities (Gould et al., 1990; Woolley & McEwen, 1992). Similarly, elevated progesterone can modulate dendritic spine densities and can suppress neuronal excitability (Woolley & McEwen, 1992). Changes in ovarian hormone concentrations can also influence receptor densities, protein expression, and ion channel activity (Hammond & Gibbs, 2011; Hazell et al., 2009).

Disruption of ovarian hormone function is associated with neuroanatomical changes and cognitive impairments (Hussain et al., 2014). In middle-age, females experience a period of hormonal dysregulation that precedes a significant decrease in the concentration of circulating ovarian hormones, resulting in reproductive senescence. During this time females may experience physiological changes and impaired cognitive ability (Hussain et al., 2014; Santoro et al., 2020). Surgical interventions (ovariectomy, oophorectomy, hysterectomy, etc.) in younger females can induce similar physiological changes and cognitive impairments (Engler-Chiurazzi et al., 2017; Hamson et al., 2016). Genetic, surgical, and aged animal models of menopause show similar behavioral and cognitive impairments as observed in humans (Hara et al., 2015; Newhouse & Dumas, 2015). These physiological and cognitive changes following ovarian hormone disruption emphasize the importance of ovarian hormone function to female cognition and behavior.

Understanding the role of ovarian hormones in neurophysiological processes and their contributions to cognitive function are important to understand the impact of ovarian hormone

loss on cognition. This thesis focusses on the neuromodulatory effects of ovarian hormones, specifically 17β-estradiol (E2) and progesterone, within the entorhinal cortex. The entorhinal cortex is a temporal lobe region that receives cortical inputs from sensory and associational regions and provides the hippocampus with the majority of its cortical inputs. The experiments presented here suggest that some of the cognitive effects of estrogen, and loss of circulating estrogens in rats, that have commonly been attributed to synaptic changes in the hippocampus, may also be due to changes in synaptic function within the entorhinal cortex.

Ovarian hormones and the reproductive cycle

Estrogens and progestogens are two families of sex hormones traditionally associated with female sexual maturation, secondary sex characteristics, and reproduction (Hamson et al., 2016). More specifically, "estrogens" and "progestogens" describe endogenous steroidal compounds, or their metabolites, that bind to estrogen and progesterone receptors, respectively. The majority of circulating ovarian hormones are produced by the ovaries, although they can also be synthesized in smaller quantities by other organs, like the brain, and types of tissues, like adipose tissue. As steroids, ovarian hormones can pass through the blood-brain barrier and influence concentrations in the brain (Engler-Chiurazzi et al., 2017). In addition to estrogens and progestogens, there are exogenous compounds that can also bind and activate estrogen and progesterone receptors. Synthetic estrogens, like ethinyl estradiol and conjugated equine estradiol, and synthetic progestogens, progestins like medroxyprogesterone acetate, may be used in birth control or hormonal therapies. Some organic compounds, like phytoestrogens found in soy-based products, can active hormone receptors as well(Domańska et al., 2021; Verma & Sharma, 2015).

There are several endogenous estrogens in the human body. Of particular interest in females are four estrogens present in adulthood: estrone, estradiol, estriol, and estetrol (Coelingh Bennink et al., 2016). Estrone and estradiol (E2) are the most abundant estrogens circulating in adulthood in a roughly 1:1 ratio (Acconcia & Marino, 2018; Engler-Chiurazzi et al., 2017). They are primarily produced by the ovaries in the periphery and can be synthesized *de novo* by various types of neurons and astrocytes in the brain (Brann et al., 2022). Estrone and E2 concentrations in rat brains also fluctuate with the estrous cycle (Kato et al., 2013). Estriol is also produced by the ovaries in small quantities and may fluctuate during the menstrual cycle; however, the

highest concentrations of estriol occur during pregnancy, when it is synthesized by the placenta (Acconcia & Marino, 2018; Coelingh Bennink et al., 2016). Estetrol is a unique estrogen, as it is only synthesized during pregnancy by the fetal liver and may be detected nine weeks into pregnancy. As blood concentrations increase in the fetus, estetrol is excreted into the amniotic fluid and diffuses into the maternal bloodstream through the placenta (Coelingh Bennink et al., 2016). Of the four estrogens, E2, is believed to be the most active form of estrogen accounting for most physiological functions associated with estrogens during the reproductive phase of life. As a result, most research focuses on E2 and its more active isoform, 17β-estradiol (Engler-Chiurazzi et al., 2017).

Like estrogens, progestogens are primarily synthesized by the ovaries, and are also synthesized by principal neurons and glial cells in the brain (Schumacher et al., 2014). There are several endogenous progestogens in humans, the majority of which are metabolites of progesterone. Apart from its precursor pregnenolone, several progesterone metabolites have been shown to affect neuronal function, including 5α-dihydroprogesterone and allopregnanolone (3α,5α-trahydroprogesterone; Guennoun, 2020; Schumacher et al., 2014). The concentration of metabolites is variable between organs and regions within the brain (González-Orozco & Camacho-Arroyo, 2019). Progesterone production is relatively low during the menstrual cycle, only increasing during the luteal phase following ovulation. Progesterone concentration significantly increases during pregnancy, peaking in the third trimester, and decreases to basal levels soon after delivery (Schindler, 2015).

Overall levels of ovarian hormones vary across the female lifespan. They contribute to fetal development *in utero*, and are elevated for a brief period following birth, but remain relatively low until mid to late childhood when puberty begins (Widholm et al., 1974; Zhang et al., 2008). As early as age 8, females begin producing increasing levels of circulating ovarian hormones until they experience menarche, their first menses. Following menarche, ovarian hormone production drastically increases for several years before stabilizing around age 16. Menarche marks the beginning of the reproductive phase of life, where pregnancy is possible (Widholm et al., 1974; Zhang et al., 2008). During this phase, estrogens and progestogens fluctuate cyclically, regulating several physiological and reproductive processes. These cyclic fluctuations are referred to as the menstrual cycle, and occur, approximately, once every 28 days. The cycle can be broken into several phases, but will be discussed here in two phases, the

follicular and the luteal phases (Barbieri, 2014; Hussain et al., 2014). The menstrual cycle begins on day 1 of the follicular phase with menses, when females shed the uterine lining, for approximately six days and the concentration of ovarian hormones are low. Following menses, the concentration of estradiol (E2), a potent estrogen, gradually increases for four days, and decreases to near menses levels during the following three days. On day 14, during ovulation, a mature ovum is released into the fallopian tube and travels to the uterus in preparation for fertilization. This marks the beginning of the luteal phase. During the luteal phase, E2 and progesterone concentrations gradually increase for seven days, peaking around day 21, and decrease to follicular concentrations by day 28 (Garcia et al., 2018; Hussain et al., 2014).

Female rodents also experience ovarian hormone fluctuations during their estrous cycle (Koebele & Bimonte-Nelson, 2016). In rats, the cycle takes an average of four days to complete, with each phase of estrous corresponding to roughly one day of the cycle. Beginning with metestrus on day 1, ovarian hormone concentrations are low, but E2 concentrations begin to increase the morning of diestrus on day 2, and continues to increase, peaking in the morning of day 3, proestrus. Around midday, E2 begins to decrease, and progesterone increases, peaking around midnight. During estrus on day 4, the concentration of both hormones return to basal levels, with E2 tapering off in the morning and progesterone around midday (Goldman et al., 2007; Hussain et al., 2014).

Cyclic changes in cognition

Variations in female behavior, cognition, neuroanatomy, and synaptic function are associated with different phases of the reproductive cycle, and changes in the relative concentrations of ovarian hormones (Bernal & Paolieri, 2021). Humans in the early follicular phase perform better than their mid-luteal counterparts on spatial and cognitive flexibility tasks, like the 3D mental rotation task (Hausmann et al., 2000; Maki et al., 2002; Peragine et al., 2022). On navigation tasks, such as the virtual Morris water maze, participants with elevated levels of estradiol perform better than those with lower levels, and take more direct paths towards the goal, and spend more time in the goal-quadrant (Patel et al., 2022). In other spatial navigation studies, participants in the mid-luteal phase demonstrate a preferential bias towards reference-based strategies to solve navigation tasks, while those in the early follicular and ovulatory phases are more likely to use response-based strategies (Hussain et al., 2016). Attention and emotional

memory recall are also increased during the mid-luteal phase (Ertman et al., 2011; Nielsen et al., 2013; Yao et al., 2022), while verbal memory is enhanced around the time of ovulation (Hussain et al., 2016). Ovarian hormone concentrations are even considered during patient treatment as they can influence treatment outcomes and efficacy. Individuals receiving fear extinction training (exposure therapy), a treatment for individuals with PTSD, have improved outcomes during their mid-luteal phase, when ovarian hormones are high (Graham & Scott, 2018; Hamson et al., 2016).

Rodents exhibit similar variations in behavior and cognition associated with changes in ovarian hormone concentrations. On spatial discrimination tasks, rats demonstrate similar shifts in preferred navigation strategies between phases. Rats in proestrus, when ovarian hormones are high, preferentially use reference-based strategies, relying on environmental cues, while those in estrus, when estrogen levels are low, often use response-based or other strategies (Korol et al., 2004; Yagi et al., 2017). On the Morris water maze, rats (Pompili et al., 2010) and meadow voles (Galea et al., 1996) with high levels of estradiol perform worse compared to low estradiol groups, requiring more time to find the escape platform. Comparatively, rats in proestrus show improved performance on object recognition and working memory tasks (van Goethem et al., 2012). In fear extinction paradigms, where a conditioned fear-response like freezing is extinguished, rats and mice in proestrus (when estrogens and progestogens are high) show reduced rates of freezing when presented with the conditioned stimulus (a tone or light) in later sessions. Comparatively, rodents in estrus show no significant change in freezing following extinction training (Graham & Scott, 2018; Milad et al., 2009).

Pharmacological manipulation of ovarian hormone concentrations also induces changes in cognition and behavior. Humans taking oral contraceptives show improved verbal memory (Mordecai et al., 2008; Peragine et al., 2020), visuospatial skills (Bianchini et al., 2018), and emotional memory (Sommer et al., 2018) during the active phase of their regimens, when estrogens are elevated, compared to naturally cycling individuals. Interestingly, individuals in the active phase, show impaired performance on other tasks, like the 3D rotation task (Peragine et al., 2022). Ovarian hormone supplementation in animal studies also show alterations in performance on cognitive and behavioral tasks (Hussain et al., 2014).

Aging, reproductive senescence, and cognitive impairment

Ovarian hormone function is not consistent across the human lifespan and significantly changes in middle-age when females begin the menopausal transition. The menopausal transition is a gradual physiological process leading to a period of reproductive senescence and low ovarian hormone production (Santoro et al., 2020). The process begins around age 40 with perimenopause, when individuals experience more irregular hormonal fluctuations and decreases in ovarian hormone production. These changes in hormonal function contribute to the dysregulation of the menstrual cycle, and the time between menses becomes irregular and often increases in duration (Hara et al., 2015). The onset and progression of perimenopause is variable and differs between individuals. For some, it begins later in age and lasts only a year; for others, perimenopause may begin at an earlier age and last several years (McNeil & Merriam, 2021). Perimenopause ends with menopause, the point at which individuals experience their last menses. Menopause typically occurs around age 50 and marks the beginning of reproductive senescent phase of life. However, as perimenopause may last for several years, and the time between menses becomes irregular, menopause is only documented after a 12-month period without menses, at which point individuals are recognized as postmenopausal (Santoro et al., 2020). Unfortunately, some individuals may experience menopause before it naturally occurs due to surgical intervention, and this is referred to as surgical menopause. Surgical removal of the ovaries, bilateral oophorectomy alone or in addition to the uterus, or hysterectomy may be necessary to treat or prevent, certain medical disorders (Koch et al., 2022; Orozco et al., 2014). Premenopausal patients that undergo these procedures experience abrupt and severe decreases in the concentration and production of ovarian hormones (Rocca et al., 2014).

The menopausal transition is associated with many symptoms, including hot flashes, cold sweats, changes in emotion regulation (with increased risk of depression), and sleep disruptions. Most individuals also report changes in cognitive function, however, research to understand the prevalence and etiology of cognitive impairment is inconsistent and, at times, contradictory (Engler-Chiurazzi et al., 2017; Newhouse & Dumas, 2015). Hormone replacement therapy (HRT) may be prescribed to help alleviate symptoms, but its ability to aid cognition is also unclear. HRT is often prescribed to patients following surgical menopause to help alleviate symptoms and to counteract potential decreases in bone density and increased risks of cardiovascular disease (McNeil & Merriam, 2021; Santoro et al., 2020). These patients also

exhibit more distinct disruptions of cognitive function, showing deficits as early as 3 months after surgery (Engler-Chiurazzi et al., 2017). Administration of HRT soon after surgery can improve and/or prevent deficits in cognitive function and, in some studies, reduce the severity of measured cognitive deficits years later (Rocca et al., 2014). Several studies have found no indications of cognitive impairment during the menopausal transition or benefit of HRT (Engler-Chiurazzi et al., 2017) . Studies based on the Women's Health Initiative Memory Study (WHIMS), a large-cohort longitudinal study, found that HRT was potentially detrimental to cognition and increased risks for stroke, certain cancers, and other medical conditions (Engler-Chiurazzi et al., 2017). The potential increase of risk factors associated with HRT as reported by WHIMS and other studies, significantly curbed its administration to perimenopausal individuals over the past 20 to 30 years (Hilakivi-Clarke et al., 2013; Newhouse & Dumas, 2015).

These studies and their disparate findings have been reevaluated and contextualized in reviews to provide a broader understanding of the complex interactions between declining ovarian hormone function, cognitive disruptions, and HRT (Engler-Chiurazzi et al., 2017; Hara et al., 2015; Newhouse & Dumas, 2015). When considering studies like WHIMS, there are potentially confounding variables that may account for the negative findings regarding the potential detriment of HRT. As previously discussed, most individuals experience perimenopause their 40s, and are postmenopausal by age 50, with dramatically reduced ovarian hormone concentrations. Both human and animal studies have suggested that prolonged periods of ovarian hormone deprivation can induce a pseudo hormone insensitivity that may occur as a result of reduced hormone receptor expression (Koebele et al., 2017; Newhouse & Dumas, 2015). This informs the "critical window hypothesis," which posits that there is a finite period after menopause in which hormone supplementation can have beneficial effects (Maki, 2006; Sherwin, 2009). The proposed length of the critical period varies between reports, lasting up to 5 years after menopause, and HRT given after this period may offer no benefit or could be detrimental (Newhouse & Dumas, 2015). WHIMS participants were significantly older than 50, at approximately 65-70 years old, and the majority of participant had never received HRT prior to WHIMS. Groups randomly assigned to receive HRT began regimens on average 10 years after the critical period (Engler-Chiurazzi et al., 2017; Hamson et al., 2016). In support of the critical window hypothesis, a cross-sectional study of adults age 60 and older, found that individuals that received HRT before age 56 showed better cognitive performance than those who never received

HRT, while those who began HRT after 56 showed the worst performance (Hara et al., 2015; MacLennan et al., 2006). Other studies have found that the severity of menopausal cognitive deficits and the efficacy of HRT are also influenced by parity (Duarte-Guterman et al., 2021; Galea et al., 2014), dementia-related genetic risk factors (Duarte-Guterman et al., 2021), lifestyle choices (Boyle et al., 2021), the type of HRT regimen used (Engler-Chiurazzi et al., 2017), the peak concentrations of endogenous ovarian hormones produced during of reproductive phase, and its overall length (Matyi et al., 2019).

Like humans, females from other mammalian species undergo hormonal transitions in middle-age leading to reproductive senescence. Species with estrous cycles, like rodents, undergo the estropausal transition. In rats, estropause occurs around 12 months of age and is preceded by the sub-fertile period, a period similar to perimenopause, beginning around 8 months of age (Cruz et al., 2017; Koebele & Bimonte-Nelson, 2016). This reduction in ovarian hormone function is associated with deficits in performance on behavioral assessments of spatial navigation and memory, object recognition memory, fear conditioned memory, and changes in metabolism (Ferreira et al., 2012; Morel et al., 2015; Zhvania et al., 2021). Due to similarities between rodents and humans, they are often used as animal models of aging to better understand ovarian hormone function. In rats, ovariectomy results in a significant reduction in peripherally synthesized hormones over a period of 1 to 2 weeks (Brann et al., 2022; Hussain et al., 2014). In contrast, the concentration of brain-derived hormones can be maintained for several weeks following ovariectomy (Kato et al., 2013). However, brain-derived estrogens appear to be insufficient to maintain optimal cognitive function alone. Ovariectomy in rodents is associated with impaired performance on spatial navigation and memory tasks (Pala et al., 2019), impaired performance on object memory tasks, increased depression-like behavior (Weiser et al., 2008; Xu et al., 2015), and impaired performance on social learning tasks (Ervin, Mulvale, et al., 2015). Many of these studies have also administered HRT to the animals, and have found that supplementation of estrogens, primarily E2, is able to ameliorate or prevent many of these impairments. Supplementation of progesterone/progestogens are also capable of exerting some benefit, although the underlying mechanisms behind this remain unclear (Barros et al., 2015).

Despite our increased understanding of the relationship between ovarian hormone function and cognition, there is still debate surrounding the efficacy of HRT. Results from the Cache County Study showed that HRT improved cognitive function in postmenopausal

individuals independent of time (Matyi et al., 2019). A different study in females aged 70 and older showed that participants with a history of HRT had greater brain volume than participants without (Boyle et al., 2021). Alternatively, a recent meta-analysis of 23 randomized control trials reported significant negative cognitive effects of HRT lasting more than 6 months in individuals 60 and older (Zhou et al., 2021). Ultimately, research to understand the effects of ovarian hormones at different phases of life, and how they interact with individual physiology in each phase, is necessary to understand how HRT can be best used to exert its benefits.

Estrogenic function in the brain

Estrogens have both genomic and non-genomic effects on neuronal function and synaptic transmission within the brain. Estrogens' effects are mediated by three types of estrogen receptors expressed throughout the brain (Hazell et al., 2009; Waters et al., 2015). Estrogen receptors α (ERα) and β (ERβ) are classical ERs that primarily act as transcription factors involved in the modulation of gene expression (Björnström & Sjöberg, 2005). In contrast, the G protein-coupled estrogen receptor-1 (GPER1) is a metabotropic receptor that modulates second messenger systems to rapidly affect synaptic transmission and neuronal function (Bean et al., 2014). The expression and distribution of each ER type varies between sexes, reproductive cycle phases, ages, brain regions, cell types, and cell regions (Marraudino et al., 2021; Oberlander & Woolley, 2016; Waters et al., 2015). Some cells may express all three ER types, while others may only express one (Vajaria & Vasudevan, 2018). Of the three ERs, GPER1 receptors comprise the majority of ERs in the brain, while ERα and ERβ are expressed to a lesser degree (Merchenthaler et al., 2004).

ERα and ERβ mediate the expression of several genes essential to neuronal function, including those associated with myelination, lipid synthesis, and metabolism (Humphreys et al., 2014; Sárvári et al., 2011). They are distributed throughout the neuron in the cytosol but are also present in the mitochondria and can be bound the cell membrane (Álvarez-Delgado et al., 2010; Levin, 2009). When activated via ligand binding, or phosphorylation by second messenger cascades, ERα and ERβ form homodimer pairs (ERα-ERα; ERβ-ERβ), or heterodimer (ERα-ERβ) pairs when expressed in the same cell. These pairs subsequently enter the nucleus and bind to estrogen response elements located on target gene promoters. Activated ERs can also independently enter the nucleus and bind to transcription factors that in turn bind to their

corresponding gene promoters (Björnström & Sjöberg, 2005; Maioli et al., 2021). Although ERα and ERβ can modulate gene expression, genomic changes require substantial amounts of time to induce observable changes in physiology, cognition, and behavior; so the genomic effects of ERα and ERβ cannot account for the more rapid and transient changes that are observed within minutes of ER activation (Erli et al., 2020; Ervin, Lymer, et al., 2015; Gabor et al., 2015).

Membrane-bound ERs can have rapid effects on second messenger systems and induce changes in neuronal function within minutes. Membrane-bound ERs are expressed throughout the cell (Hazell et al., 2009; Waters et al., 2015), and can induce rapid functional changes in ion channel permeability (Foy et al., 1999), second messenger cascades (Taxier et al., 2020), postsynaptic receptor densities (Mukherjee et al., 2017), dendritic spine densities (Woolley & McEwen, 1993), and the presynaptic release of neurotransmitters (Tabatadze et al., 2015). GPER1 is the most common type of mER, although isoforms of ERα ($ER\alpha_{36}$) and ERβ are also membrane-bound in some cells (Vrtačnik et al., 2014; Zhang et al., 2012). As a G protein-coupled receptor, the effects of GPER1 activation depend on the G protein subunit coupled with it. GPER1 can couple with both $G\alpha_s$ and $G\alpha_{i/o}$ subunits to affect intracellular cAMP, IP3, Erk, PLC, and MAPK/AKT cascades in hippocampal and hypothalamic cells (Benmansour et al., 2016; Vajaria & Vasudevan, 2018). In the hippocampus, GPER1 activation can increase synaptic transmission via suppression of inhibitory transmission (Mukherjee et al., 2017; Tabatadze et al., 2015). It can also facilitate excitation via modulation of ERK (Kumar et al., 2015) and adenylyl cyclase and cAMP signaling (Alexander et al., 2017; Lu & Herndon, 2017). Furthermore, GPER1 receptors can influence the activity of other G protein-coupled receptors like 5-HT1_A, corticotropin and progestogen receptors (Akama et al., 2013). Membrane-bound ERα and ERβ can also rapidly influence synaptic transmission and cell excitability (Clemens et al., 2019; Kumar et al., 2015; Tanaka & Sokabe, 2013). ERs can be bound to the organelles of certain cells, and GPER1 receptors have been observed bound to the endoplasmic reticulum and Golgi apparatus of hippocampal pyramidal cells (Matsuda et al., 2008; Revankar et al., 2005). The endoplasmic reticulum is implicated in lipid metabolism, protein synthesis, and calcium-signaling as an intracellular store of Ca^{2+} (Schwarz & Blower, 2016). Activation of endoplasmic reticulum-bound GPER1 receptors induces Ca^{2+} release from internal stores, to influence calcium-signaling pathways and second messenger cascades, including TrkA, ERK, and PI3K (Benmansour et al., 2016; Fan et al., 2010; Gingerich et al., 2010; Kumar et al., 2015).

Activation of mitochondria-bound ERα (Bauzá-Thorbrügge et al., 2019) and ERβ (Uddin et al., 2020) can contribute to the maintenance of cellular health and homeostasis via metabolism of proinflammatory cytokines that can disrupt cellular function and synaptic transmission.

Disruption or loss of estrogenic function related to age, pharmacological, or surgical manipulation can have marked effects on gene expression, synaptic function, and neuroanatomy throughout the brain (Humphreys et al., 2014). Bilateral ovariectomy reduces the expression of all ERs in various regions including the hippocampus, entorhinal cortex, basal forebrain, and prefrontal cortex (Wang et al., 2018). There are significant reductions in mitochondrial function and an increase in proinflammatory cytokines (W. Zhao et al., 2021). Hippocampal dendritic spine densities fluctuate with the estrous cycle and are significantly reduced following ovariectomy (Woolley & McEwen, 1993). There are also marked reductions in synaptic excitability in the hippocampus, changes in ionotropic receptor densities, and changes in calcium signaling (Hammond et al., 2011; Mukherjee et al., 2017; Oberlander & Woolley, 2016). These changes in synaptic function may be due to changes in receptor expression (Blurton-Jones & Tuszynski, 2006; Vaucher et al., 2002), receptor localization (Mukherjee et al., 2017), or to other functional mechanisms of synaptic transmission (Tabatadze et al., 2015). Estrogens are also implicated in the function of other neurotransmitter systems, and estrogenic disruption adversely affects dopaminergic (Almey et al., 2015; Espinosa & Curtis, 2018), serotonergic (Long et al., 2019), GABAergic (Blurton-Jones & Tuszynski, 2006; Vaucher et al., 2002), and cholinergic systems (Gibbs, 1997; Hammond & Gibbs, 2011).

Estrogens and cholinergic function

The cholinergic system is important for cognitive function and has significant roles in attention, sensory information processing, reward, and addiction, and learning and memory (Hasselmo, 2006). Cholinergic function is also implicated in spatial navigation and object recognition (Solari & Hangya, 2018). The basal forebrain houses several nuclei that provide the majority of cholinergic projections to the cortex and the hippocampal formation (Woolf & Butcher, 2011). The hippocampal formation receives cholinergic projections from the medial septum, nucleus basalis of Meynert, and the diagonal band of Broca. The entorhinal cortex also receives strong cholinergic projections from the medial septum and the diagonal band of Broca (Kondo & Zaborszky, 2016; Woolf & Butcher, 2011). Cholinergic inputs to the entorhinal cortex

and hippocampus promote synchronization of neurons by enhancing theta and gamma EEG activities (Hamam et al., 2007; Klink & Alonso, 1997), result in a suppression of excitatory glutamatergic synaptic transmission (Barrett & Chapman, 2013), and also increase the excitability of neurons by depolarizing resting membrane potential (Hamam et al., 2007; Klink & Alonso, 1997).

Estrogens are thought to contribute to cognitive function by maintaining and enhancing cholinergic modulation of excitatory synapses and neuronal excitability in the hippocampal region. It is well established that cholinergic projections to the entorhinal cortex and hippocampus play an important role in cognitive functions (Solari & Hangya, 2018), and evidence suggests that the effect of estrogens on cognitive function may be mediated in part by their ability to modulate cholinergic function in the basal forebrain and cholinergic transmission in the cortex and hippocampal formation (Hammond & Gibbs, 2011; Horvath et al., 2002). Up to 99% of cholinergic neurons in the basal forebrain express GPER1 receptors (Gibbs et al., 2014; Hammond et al., 2011) and show changes in soma characteristics and concentration of cholinergic proteins when comparing phases of estrous (Gibbs, 1996; Luine, 1985). Because estrogen promotes the function of basal forebrain cholinergic neurons, cognitive functions that vary with the estrous cycle may do so because of changes in cholinergic transmission associated with the menstrual/estrus cycle.

Consistent with the idea that E2 promotes the function of basal forebrain cholinergic neurons, loss of estrogens in rats following ovariectomy can disrupt cholinergic transmission. Ovariectomy significantly reduces the concentration of these proteins, but supplementation with E2 or ER-specific ligands can prevent or remedy these reductions on par with intact animals (Hammond & Gibbs, 2011). Ovariectomy also impairs cholinergic transmission to the hippocampus, due in part to a reduction in the number of cholinergic neurons in the basal forebrain and projections to the hippocampus (Gibbs et al., 2014; Hammond et al., 2011). Estrogen supplementation can ameliorate some of these effects, but the degree of benefit is dependent upon the duration of deprivation (Gibbs, 2000; Tinkler et al., 2004). One theory has suggested that many of the alterations in cognition following ovariectomy may be due to reductions in cholinergic transmission (Newhouse & Dumas, 2015) and supporting data strongly suggest that ovariectomy results in cognitive changes in part through a reduction in cholinergic transmission in the hippocampus and other cortical regions (Hammond & Gibbs, 2011).

The hypothesis that loss of estrogens disrupts cognition in part through a reduction in the function of basal forebrain cholinergic neurons is also consistent with findings that there may be a critical window, around time of menopause, in which hormone replacement therapy may have lasting cognitive benefits (Newhouse & Dumas, 2015). It has been suggested that delaying HRT for a long period following menopause can reduce the potential effectiveness of HRT in enhancing cognition because of a reduction in estrogen receptors following menopause (Engler-Chiurazzi et al., 2017) . However, it is also possible that HRT may decline in effectiveness because of lasting changes in cholinergic neurons after menopause (Brinton, 2004; Harman et al., 2004; Newhouse & Dumas, 2015). Central to this idea is that HRT can promote the maintenance of cognitive function following menopause through the effects of estrogens on cholinergic neurons.

The entorhinal cortex

The entorhinal cortex is situated in the medial temporal lobe and contributes to spatial mapping, navigation, object recognition, and olfactory processes. It receives sensorimotor and associational information from other cortical regions and sends its major output projections to the hippocampus (Burwell, 2000; Knierim et al., 2013). While the entorhinal cortex provides the hippocampus with the majority of its cortical inputs, it also receives projections from the hippocampus. The entorhinal cortex is therefore a component of a parahippocampal-hippocampal network in which information can be transmitted bidirectionally between the hippocampus and widespread cortical regions (Nilssen et al., 2019). This network is believed to be a major region that contributes to cognitive processes that affect the salience of sensory stimuli, working memory, object recognition, and declarative memory (Burwell, 2000; Deshmukh & Knierim, 2011; Hafting et al., 2005; Hasselmo, 2006; Knierim et al., 2013) .

In rats, the entorhinal cortex can be divided into two sub-regions based upon their contributions to cognitive processes, cortical projections, and architecture. The information processed in the medial and lateral entorhinal cortex is believed to be integrated through interconnections between the LEC and MEC, and this is thought to provide highly processed associational cortex input to the hippocampus (Eichenbaum et al., 2007; Nilssen et al., 2019). The medial entorhinal cortex (MEC) contributes to spatial mapping, navigation, and contextual memory. A role for the MEC in spatial processing was demonstrated with the discovery of grid

cells in the MEC (Moser et al., 2021). In contrast to place cells in the hippocampus, which fire when animals are within a restricted area of the environment, grid cells in the MEC show maximal firing in areas that correspond to the vertices of a hexagonal grid (Sanders et al., 2015). The region also houses cells that respond to changes in speed, similar to speed cells in the hippocampus (Ye et al., 2018). This has been proposed to contribute to spatial navigational processes involved in dead reckoning, in which navigation is based on egocentric factors including head direction and locomotor activity (Moser et al., 2021) . Furthermore, lesions to the MEC can impair the acquisition of, and performance on, spatial navigation and memory tasks like the Morris water maze (Hales et al., 2018). Lesions can also impair context- and stimulus-dependent fear-conditioned responses and memories (Hales et al., 2018).

In contrast, the lateral entorhinal cortex (LEC) is believed to primarily contribute to non-spatial contextual memory and working memory. Studies of the LEC provide evidence of its involvement in object recognition, object-position memory (van Cauter et al., 2013; Kuruvilla & Ainge, 2017), social memory (Leung et al., 2018; Lopez-Rojas et al., 2022), and olfaction (Hutter & Chapman, 2013; Leitner et al., 2016). Like the MEC, the LEC has spatially responsive cells that correspond to the placement of objects in each environment (Deshmukh & Knierim, 2011). Lesions to the LEC do not impair performance and acquisition of spatial tasks, like the Morris water maze (Citation, or link to next sentence). However, lesions can impair rats' performance on spatial and nonspatial object memory tasks (van Cauter et al., 2013; Rodo et al., 2017). The differences in the behavioral and cognitive contributions of the lateral and medial regions of the entorhinal cortex suggest that the two regions provide complementary streams of highly processed sensory information to the hippocampus.

Laminar and cellular organization

The distinctions between the medial and lateral entorhinal cortex can also be seen in their cellular morphology, physiology and organization, and their internal and external functional connectivity. Like other cortical regions, the entorhinal cortex is organized into six layers that can be divided into the superficial layers (LI, LII, & LIII) and the deep layers (LV & LIV). Layer IV is a thin layer that lacks cell bodies and separates the superficial and deep layers. The superficial layers receive the majority of the cortical projections to the entorhinal cortex, although projections to the deep layers have also been observed (Nilssen et al., 2019).

Projections to the superficial layers of the entorhinal cortex are region-specific and differ between the lateral and medial regions. Superficial layers of the LEC receive major projections from the piriform (primary olfactory) cortex, perirhinal cortex, olfactory bulbs, and prefrontal cortex, with weaker inputs from the postrhinal cortex. The LEC also receives projections from the olfactory bulbs and the prefrontal cortex to the deep layers. MEC superficial layers receive major inputs from the perirhinal and postrhinal cortex, the presubiculum, the parasubiculum, and the retrosplenial cortex. The deep layers also receive projections from the retrosplenial cortex and parasubiculum (Nilssen et al., 2019). These differences in projections to the LEC and MEC support the differences in contribution to cognition and behavior previously mentioned.

The types of neurons present in the entorhinal cortex, their organization, and location, also differ between the LEC and MEC. Generally, LI of the entorhinal cortex primarily contains axonal inputs from other cortical regions, dendrites of entorhinal neurons, and lacks the presence of cell bodies, except for some GABAergic interneurons. Layer II of the MEC contains primarily stellate and pyramidal neurons, while layer II of the LEC contains primarily fan and pyramidal neurons. Layer III of the MEC and LEC contains mainly spiny and non-spiny pyramidal cells and multipolar cells. Layer V in both the LEC and MEC contain predominantly pyramidal and multipolar cells that vary in morphology, size, and dendritic arbor. The organization and size of pyramidal cells create two LV sublayers: LVa and LVb. Pyramidal cells in LVa are relatively large, vary in soma shape, and are spread unevenly between the LEC and MEC. Comparatively, pyramidal cells in LVb are more consistent in size and shape and are more densely packed than in LVa (Nilssen et al., 2019).

In addition to their location and morphology, entorhinal cortex cells can be classified by their protein expression, connectivity, and physiological characteristics. Entorhinal cortex principal neurons can be classified based on their expression of calbindin (CB) and reelin proteins (Witter et al., 2017). Reelin is a large extracellular protein that has different functions across the lifespan. During the early stages of gestation, reelin contributes to the organization and development of the cortex, and facilitates the migration of young neurons from the ventricular zone to the developing cortex. At later stages of gestation, reelin promotes dendritic growth and branching in the cortex and hippocampus. Postnatally, and into adulthood, reelin is associated with synaptogenesis and the regulation of synaptic function. Reelin activity can modulate dendritic spine growth, synaptogenesis, AMPAR- and NMDAR- mediated transmission, and

Ca^{2+} signaling and second messenger cascades (Jossin, 2020). Calbindin is a calcium-binding protein that acts as a calcium buffer and binds to calcium to regulate its activity and maintain homeostatic states (Yáñez et al., 2012). It is expressed in both pyramidal and non-pyramidal cells, of which 73% also express GABA (DeFelipe, 1993, 1997). Layer II MEC stellate cells express RE, while pyramidal cells express CB. Interestingly, LII MEC principal neurons cluster together to form islands of RE-positive stellate cells and islands of CB-positive pyramidal cells. Principal neurons in LEC LII follow a similar pattern of protein expression, in which pyramidal cells express CB and fan cells express RE. Layer II LEC principal neurons also cluster together with similar cells, forming two sub-layers; LIIa contains RE-positive fan cells and LIIb contains CB-positive pyramidal cells. Both MEC and LEC LIII have more uniform distributions of principal and multipolar neurons that express reelin (Witter et al., 2017) .

Like principal neurons, cortical interneurons can be classified by their protein expression. Cortical interneurons can be classified into three broad classes: parvalbumin (PV), somatostatin, and 5-$HT3_AR$ proteins (Lim et al., 2018). Parvalbumin is another calcium-binding protein expressed in non-pyramidal cells, and it can overlap with CB expression. In the rat cortex, GABAergic interneurons are often PV-positive (DeFelipe, 1993, 1997; Yáñez et al., 2012). Somatostatin is a neuropeptide associated with cortical inhibition (Song et al., 2021), and 5-$HT3_ARs$ are ionotropic serotonin receptors (Huang et al., 2016). Although cells within each class share many characteristics, sub-populations of neurons within each class can exhibit characteristics from the other classes (Lim et al., 2018; Song et al., 2021). The distribution and organization of entorhinal interneurons varies between the lateral and medial regions, and between cortical layers. Parvalbumin-positive interneurons constitute approximately half of the interneurons in the entorhinal cortex, and interneurons in LII of the MEC are predominantly PV-positive. Comparatively, LEC LII, especially LIIa, shows a limited number of PV-positive interneurons, and has predominantly 5-$HT3_AR$-positive interneurons present. Somatostatin-positive interneurons appear to be evenly spread throughout the entorhinal cortex and are co-localized with GABAergic markers (Witter et al., 2017). The majority of PV- and somatostatin-positive interneurons in the entorhinal cortex also express GABA (Kecskés et al., 2020); however, some interneurons, including 5-$HT3_AR$-positive cells, may also serve as modulatory neurons that may release acetylcholine, dopamine, or other neurotransmitters (Lim et al., 2018).

GABAergic interneurons strongly affect the function of principal neurons and can mediate interactions between principal neurons. Distinct groups of inhibitory interneurons mediate interactions between stellate cells and between pyramidal cells in LII. Functional studies reveal that MEC stellate cells show minimal interactions via direct excitatory synaptic connections, but rather communicate via PV-positive GABAergic interneurons that mediate disynaptic inhibitory currents (Hafting et al., 2008). MEC pyramidal cells, in contrast, interact through 5-HT3$_A$R-positive interneurons (Hafting et al., 2008). The resulting mutual inhibition among principal neurons, combined with the separate interneuron populations, is thought to contribute to the mechanisms that organize and maintain grid cell firing associated with the cortical mapping of the environment (Hafting et al., 2008). LEC fan cells demonstrate a similar network of connectivity with one another, forming disynaptic inhibitory connections using interneurons. Nilssen et al. (2018) found that LII fan cells, especially RE-positive cells in LIIa, mainly create disynaptic inhibitory connections with fast spiking and non-fast spiking interneurons to communicate with other fan cells. Unfortunately, the fast spiking and non-fast spiking cells are not clearly associated with any specific class of protein expression. However, many fast-spiking cells were found to be PV-positive. The segregation of MEC pyramidal and stellate neurons based on inhibitory interactions suggests that stellate and pyramidal neurons may provide distinct forms of information to the dentate gyrus and hippocampus (Lipton & Eichenbaum, 2008). In comparison to LII, layers III-VI demonstrate more direct excitatory synaptic connections between principal neurons in both LEC and MEC (Nilssen et al., 2019) .

Neurons in the superficial layers provide the main cortical inputs to the hippocampus. Layer II RE-positive principal neurons in both the MEC and LEC project to the CA2, CA3, and dentate gyrus regions, while CB-positive cells project to the CA1 region, subiculum, and the contralateral entorhinal cortex (Nilssen et al., 2019). Layer III neurons mirror CB-positive neurons from LII, and project to the CA1 region, subiculum, and the contralateral entorhinal cortex. Layer III projections to the hippocampus are reciprocated by inputs to the deep layers of the entorhinal cortex from the CA1 and subiculum. Layer Vb neurons receive projections from the CA1 and subiculum and, in turn, send projections to LVa and the superficial layers. Layer Va neurons send projections to the superficial layers and to external cortical and subcortical structures (Ohara et al., 2018; Witter et al., 2017). Layer V pyramidal cells also share axon collaterals between the LEC and MEC, and this is thought to be an important point of

communication between the two regions (Van Cauter et al., 2013; Tahvildari & Alonso, 2005; Witter et al., 2017). The projections from the deep layers to the superficial layers of the entorhinal cortex provide a mechanism with which hippocampal activity may affect LII and LIII neurons that provide inputs to the hippocampus. These may serve as a feedback network with which the hippocampus and entorhinal cortex can reinforce and/or update new memories prior to their consolidation and storage (Ohara et al., 2018; Witter et al., 2017).

Estrogens in the entorhinal cortex

Multiple brain regions including the hippocampus, somatosensory cortex, prefrontal cortex, basal forebrain, and striatum have been shown to be influenced by the estrous cycle, and changes in cognition associated with the estrus cycle and manipulation of ovarian hormones has been attributed to neurophysiological changes in these regions. The entorhinal cortex contributes to spatial memory, navigation, object memory, and olfaction (Knierim et al., 2013), and it also provides the hippocampus with most of its cortical inputs (Nilssen et al., 2019). Despite the central role of the entorhinal cortex within the medial temporal lobe, it is unclear how estrogens may modulate cognitive function by influencing synaptic and neuronal functions within the entorhinal cortex.

All three ER types are expressed in the entorhinal cortex (Kritzer, 2002), suggesting that estrogens likely modulate entorhinal cortex function. GPER1 expression is most abundant, and ERβ has much greater expression than ERα, which is relatively sparse (Hazell et al., 2009; Merchenthaler et al., 2004; Shughrue et al., 1997). ERα expression has been observed in layer V non-pyramidal cells that do not express parvalbumin (PV) or calbindin (CB; Kritzer, 2002). ERβ expression is much greater than ERα, and has been localized to non-pyramidal cells in layers III-VI that express PV and CB separately or concurrently (Kritzer, 2002) . This suggests that ERβ expression occurs in GABAergic interneurons and can influence synaptic transmission via modulation of inhibition (Clemens et al., 2019; Kritzer, 2002). Furthermore, the modulation of inhibition may be specific to cell sub-type and region as there are differences in ER expression between the lateral and medial entorhinal cortex. Kritzer (2002) found that all ERβ expressing cells are positive for CB; however, approximately 50% of ERβ cells in the lateral entorhinal cortex express PV, but none of the ERβ cells in the medial entorhinal cortex do so. This suggests that ERβ may contribute to synaptic transmission via modulation of distinct types of inhibitory

GABAergic interneurons in the lateral vs. medial entorhinal cortex. ERα expression is comparatively localized to the deep layers, layers V and VI, of the entorhinal cortex and was not co-localized with PV or CB. This suggests that the effects of ERα activation may be associated with modulation of excitatory principal neurons (Kritzer, 2002). Of course, as these receptors are nuclear ERs, their presence in these cells may not rapidly modulate synaptic function or transmission, but rather, affect gene expression. GPER1 receptor expression has been observed throughout the entorhinal cortex (Hazell et al., 2009). Unlike ERα and ERβ, GPER1 expression has not been localized to a specific subtype of cells in the entorhinal cortex. In the hippocampus GPER1 expression has been observed in pyramidal cells, glia, dendrites, axons, and organelles (Revankar et al., 2005; Waters et al., 2015). As GPER1 is primarily a mER, it may also rapidly enhance synaptic transmission via increases in synaptic excitability or changes in calcium conductance (Hadjimarkou & Vasudevan, 2018; Oberlander & Woolley, 2016).

Aside from studies indicating the expression and localization of ERs, little is known of the effects of estrogens on synaptic function in the entorhinal cortex. Few studies have explored the roles and contributions of estrogens in the region. Ovariectomized rats with low estrogen replacement showed enhanced performance on the novel object preference task when acute high doses of estradiol benzoate or intra-perirhinal/entorhinal cortex infusions were administered immediately before or after the familiarization phase. Interestingly, these same infusions impaired performance on the delayed non-matching to sample task (Gervais et al., 2013). E2 supplementation to supraphysiological levels increases the severity and occurrence of seizure-like activity in the entorhinal cortex and hippocampus (CA3 and DG) of intact female rats in an epilepsy model (Velíšková & Velíšek, 2013). E2 supplementation following excitotoxic lesions to the lateral entorhinal cortex increases axonal sprouting from the hippocampus towards the superficial layers of the entorhinal cortex in intact female rats. Ovariectomized rats showed significantly reduced amounts of sprouting following lesion and treatment, as indicated by decreased synaptophysin and AChE staining in the DG (Kadish & Groen, 2002). Together, these findings indicate that estrogens within the entorhinal cortex can affect cognitive processes involved in learning and memory, but the cellular and synaptic mechanisms remain unclear.

This thesis aims to better characterize the role of estrogens in the entorhinal cortex and how they contribute to synaptic function in the region. Improvements in cognitive function associated with estrogen have usually been attributed to cellular and synaptic alterations in the

hippocampus, but because the entorhinal cortex is so closely linked with hippocampal function, it is also possible that estrogen may promote cognitive processes through effects in the entorhinal cortex. In the hippocampus, it is well established that E2 can cause rapid enhancements in the strength of excitatory synaptic transmission (Oberlander & Woolley, 2016), and it is possible that this contributes to estrogen-dependent improvements in cognitive performance on hippocampal-dependent tasks. Similarly, loss of estrogens is thought to interfere with cognition through changes in cholinergic transmission and excitatory synapses within the hippocampus (Hammond & Gibbs, 2011), but it is not clear to what extent loss of estrogen may lead to similar changes in the entorhinal cortex.

Experimental chapter summaries

The purpose of this thesis is to better understand the contributions of estrogens to the maintenance and modulation of synaptic function in the entorhinal cortex. Chapter 2 (Batallán Burrowes et al., 2021) assessed the rapid functional effects of acute estrogen application on synaptic transmission in the entorhinal cortex, using field excitatory postsynaptic potentials (fEPSPs) recorded in brain slices collected from ovariectomized female rats receiving low dose estrogen replacement. Application of 17β-estradiol (E2) for 20 minutes reversibly increased fEPSP amplitudes in layer II of the entorhinal cortex, consistent with previous studies conducted in the hippocampus (Kumar et al., 2015; Oberlander & Woolley, 2016). However, application of progesterone or allopregnanolone did not result in any significant changes to fEPSP amplitudes. Application of specific estrogen receptor agonists was then used to assess the contribution of each receptor subtype to the E2-induced increase in synaptic strength. Application of the ERα specific agonist PPT and the ERβ specific agonist DPN did not significantly increase fEPSP amplitudes. These results suggest that the two receptor subtypes do not significantly contribute to the rapid enhancement in fEPSPs that was observed during E2 application. Application of the GPER1 receptor agonist G1, however, did increase fEPSP amplitudes, similar to enhancements observed with E2. Further, the ability of E2 to increase fEPSP amplitudes was blocked in the presence of the specific GPER1 antagonist G15. In contrast to findings in the hippocampus, in which facilitation of EPSPs is associated with activation of ERa ERβ or GPER1 receptors (Kumar et al., 2015; Oberlander & Woolley, 2016) , these findings show that activation of

GPER1 receptors is important for the rapid enhancement of excitatory transmission in the entorhinal cortex induced by E2.

The findings of Chapter 2 determined that E2 rapidly and reversibly facilitates the strength of mixed excitatory synaptic field potentials in populations of entorhinal cortex cells, but these changes could have been due to effects of E2 on AMPA or NMDA glutamate receptors and could also have been due to a reduction in the strength of synaptic inhibition. Chapter 3 therefore explored the effects of estrogen on layer II or III principal neurons in the entorhinal cortex using whole-cell patch-clamp recordings of excitatory NMDA and AMPA receptor-mediated currents, as well as isolated inhibitory $GABA_A$ receptor-mediated currents. Using intracellular recordings under voltage-clamp conditions, acute application of E2 did not significantly enhance pharmacologically isolated AMPA or NMDA receptor-mediated currents. However, E2 resulted in a reversable reduction in inhibitory $GABA_A$ receptor-mediated currents. This reduction in synaptic inhibition is therefore a likely mechanism through which E2 resulted in the facilitation of mixed field excitatory postsynaptic potentials reported in Chapter 2. Because the facilitation of fEPSPs observed in Chapter 2 was dependent on activation of GPER1 receptors, it is likely that the reduction in the amplitudes of IPSCs was dependent on GPER1 receptors that reduce GABA transmission through either presynaptic or postsynaptic mechanisms (Blurton-Jones & Tuszynski, 2002; Mukherjee et al., 2017; Tabatadze et al., 2015).

In contrast to the rapid functional effects discussed in Chapters 2 and 3, Chapter 4 explored the delayed effects of prolonged estrogen deprivation on synaptic transmission in the entorhinal cortex. The experiments discussed in Chapter 4 explore how the prolonged deprivation of circulating estrogens affects cholinergic transmission and the modulation of excitatory transmission in the entorhinal cortex (Batallán Burrowes et al., 2022). Western blot analyses were used to assess changes in cholinergic protein expression two weeks following ovariectomy in groups of Long-Evans rats that either received a sham surgery, were ovariectomized, or were ovariectomized with replacement of E2. Acetylcholinesterase is an enzyme responsible for the degradation of acetylcholine, and was used as marker of cholinergic projections (Gibbs et al., 1994). Vesicular acetylcholine transporter is a protein associated with the packaging of acetylcholine into vesicles and was used as marker of cholinergic terminal projections in the entorhinal cortex (Bennett et al., 2009). The muscarinic M_1 receptor, which is present on both postsynaptic neurons and glutamate terminals, was also quantified because of its

involvement in the cholinergic reduction of glutamate release in the entorhinal cortex (Barrett & Chapman, 2013). There were no significant differences in VAChT expression between the three groups, which suggests that the density of cholinergic terminals in the entorhinal cortex remained unchanged following prolonged deprivation of estrogens. However, there were significantly lower levels of AChE and M_1 receptor protein in ovariectomized rats compared to sham rats, and these reductions were prevented in rats treated with E2 following ovariectomy. There were no differences between the sham and E2 treated groups, or between the medial and lateral regions of the entorhinal cortex. These results indicate that loss of circulating estrogens can reduce the expression of cholinergic proteins, potentially disrupting synaptic function and transmission, and that low levels of E2 supplementation immediately after ovariectomy can prevent these changes.

Chapter 4 also assessed the effects of ovariectomy on the capabilities of cholinergic inputs to modulate excitatory glutamatergic transmission in the entorhinal cortex. Activation of presynaptic M_1 receptors reduces the release of glutamate in the entorhinal cortex and reduces the strength of synaptic transmission (Barrett & Chapman, 2013; Heys et al., 2012). Eserine, an acetylcholinesterase inhibitor, was used to prevent the breakdown of endogenous ACh, extending its presence in the synapse, and reducing the strength of excitatory transmission (Barrett & Chapman, 2013; Mans et al., 2014). Electrically evoked postsynaptic field potentials (fEPSPs) amplitudes were measured to assess changes in the efficacy of cholinergic inputs to modulate excitatory transmission following ovariectomy. In comparison to baseline responses, fEPSP amplitudes were significantly reduced in both ovariectomized and intact female rats following eserine application. However, the magnitude of decrease was significantly reduced in recordings from ovariectomized rats. This reduction suggests an impairment of the cholinergic system occurs following ovariectomy, and this interpretation is supported by observed reductions in acetylcholinesterase and M_1 receptor protein using Western blots. Together, these findings suggest that ovariectomy impairs cholinergic function within the entorhinal cortex, and suggests that some of the cognitive effects associated with loss of estrogens (Hammond et al., 2011) may be due to alterations in the modulatory effect of estrogen on excitatory transmission in the entorhinal cortex.

CHAPTER 2

G PROTEIN-COUPLED ESTROGEN RECEPTOR-1 ENHANCES EXCITATORY SYNAPTIC RESPONSES IN THE ENTORHINAL CORTEX

ABSTRACT

Activation of estrogen receptors is thought to modulate cognitive function in the hippocampus, prefrontal cortex, and striatum by affecting both excitatory and inhibitory synaptic transmission. The entorhinal cortex is a major source of cortical sensory and associational input to the hippocampus, but it is unclear if either estrogens or progestogens may modulate cognitive function through effects on synaptic transmission in the entorhinal cortex. The present study assessed the effects of brief application of either 17-β estradiol (E2) or progesterone on excitatory glutamatergic synaptic transmission in the female rat entorhinal cortex in vitro. Rats were ovariectomized on postnatal day (PD) 63 and also received subdermal E2 implants to maintain constant low levels of circulating E2 on par with estrus. Electrophysiological recordings from brain slices were obtained between PD70 and PD86, and field excitatory postsynaptic potentials (fEPSP) reflecting activation of the superficial layers of the entorhinal cortex were evoked by stimulation of layer I afferents. Application of E2 (10 nM) for 20 min resulted in a small increase in the ampliutude of fEPSPs that reversed during the 30-min washout period. Application of the ERα agonist PPT (100 nM) or the β agonist DPN (1 μM) did not significantly affect synaptic responses. However, application of the G protein-coupled estrogen receptor -1 (GPER1) agonist G1 (100 nM) induced a reversible increase in fEPSP amplitude similar to that induced by E2. Further, the potentiation of responses induced by G1 was blocked by the GPER1 antagonist G15 (1 μM). Application of progesterone (100 nM) or its metabolite allopregnanolone (1 μM) did not significantly affect synaptic responses. The potentiation of synaptic transmission in the entorhinal cortex induced by activation of GPER1 receptors may contribute to the modulation of cognitive function in female rats.

Estrogens and progesterone are ovarian hormones that support cognitive function through actions on brain regions including the hippocampus, cortex, and striatum (Frick & Kim, 2018; Henderson, 2018; Rossetti et al., 2016). Estrogen is also synthesized and released within the brain in both males and females where it modulates neuronal function and synaptic transmission (Finney et al., 2020; Oberlander & Woolley, 2016; Hojo et al., 2008). Estrogen facilitates excitatory synaptic transmission in the hippocampus, and this may contribute to hippocampus-dependent enhancements in learning and cognitive function (Hussain et al., 2016; Hadjimarkou & Vasudevan, 2018; Sellers et al., 2015). The entorhinal cortex, which receives inputs from cortical sensory and associational areas, provides the hippocampal formation with the majority of its cortical inputs (Burwell, 2000; Witter et al., 2017). The entorhinal cortex expresses minimal estrogen receptor (ER) α (Kritzer et al., 2002; Merchenthaler et al., 2004), but both ERβ, and G protein-coupled estrogen receptor-1 (GPER1) are highly expressed in the entorhinal cortex (Shima et al., 2003; Kritzer et al., 2002; Hazell et al., 2009). This suggests that the cognitive effects of estrogen may be driven by the modulation of synaptic function in both the hippocampus and entorhinal cortex.

Variations in circulating estrogen levels are able to improve cognitive performance and modulate shifts in the use of learning and memory strategies (Hussain et al., 2014; Hamson et al., 2016; Finney et al., 2020). Women taking oral contraceptives exhibit improvements in verbal memory during the active phase of their regimen, when estrogens are elevated (Mordecai et al., 2008). Naturally-cycling women in the luteal phase of the menstrual cycle demonstrate improved attention and increased retention of emotional memories (Ertman et al., 2011). Additionally, women in the mid-luteal phase, when estrogens are elevated and progesterone levels are highest, are more likely to use spatial learning strategies to solve navigation tasks. Comparatively, women in the early follicular phase, when both estrogens and progesterone are low, are more likely to use response-based strategies. The use of spatial-based strategies is believed to rely on hippocampal function, while response-based strategies rely on striatal function (Hussain et al., 2014, 2016). Similarly, administration of ovarian hormones in animal models also results in shifts in strategy on spatial tasks, in which elevated levels of 17-β estradiol (E2) promote hippocampus-dependent spatial strategies (Korol & Kolo, 2002). In spatial discrimination tasks, response-based strategies that involve the striatum are used more during estrus when estrogen

levels are low, and place-based strategies which depend on hippocampal function are preferred during proestrus when estrogens are elevated (Korol, et al., 2004).

The entorhinal cortex is known to play a major role in object memory, spatial processing and navigation (Sanders et al., 2015) and the effects of estrogens in the entorhinal cortex may affect the use of cognitive strategies based on the functions of the medial and lateral entorhinal cortex. The medial entorhinal cortex receives inputs from perirhinal and postrhinal cortex and plays a prominent role in the spatial memory and navigation (Burwell, 2000; Hafting et al., 2005), and the lateral entorhinal cortex receives major inputs from piriform and perirhinal cortex and contributes to the memory for objects and their spatial location (Knierim et al., 2013; Nilssen et al., 2019). The medial and lateral entorhinal cortex are closely interrelated functionally (Nilssen et al., 2019), but it is possible that increases in estrogen in the medial entorhinal cortex may promote spatial functions associated with path integration and navigation based on internal spatial representations, and that increases in the lateral entorhinal cortex may promote object identification memory and its association with spatial location (Deshmukh & Knierim, 2011; Knierim et al., 2013; Van Cauter et al., 2013).

Estrogens can facilitate excitatory synaptic transmission in the CA1 region by increasing dendritic spine density and can also induce rapid increases in excitatory synaptic strength (Finney et al., 2020; Sellers et al., 2015). Hippocampal spine densities vary with the estrous cycle and are greatest during proestrus when levels of estrogens and progesterone are high (Woolley et al., 1990), and the decline in dendritic spine density that occurs following ovariectomy can be prevented by estrogen replacement (Gould et al., 1990; Woolley & McEwen, 1993). The increases in spine density in the CA1 region induced by estrogens is thought to result from a reduction of GABA release and reduced $GABA_A$ receptors at the synapse (Tabatadze et al., 2015; Murphy et al., 1998; Mukherjee et al., 2017), and the disinhibition of principal neurons that results in activation-dependent increases in spines (Woolley et al., 1990).

Estrogen application can also result in rapid increases in synaptic strength in the CA1 region in vitro (Smejkalova & Woolley, 2010; Rudick & Woolley, 2001; Kumar et al., 2015). Application of estrogens in hippocampal slices increases synaptic responses by approximately 10 to 20 % (Oberlander & Woolley, 2016; Kim et al., 2006) although this effect does not occur in all of the neurons or slices tested (Sharrow et al., 2002; Rudick & Woolley, 2001; Smejkalova & Woolley, 2010). Activation of estrogen receptors can also rapidly increase glutamate release and

increase glutamate receptor sensitivity (Oberlander & Woolley, 2016). Rapid increases in synaptic responses have been linked to ERα (Tanaka et al., 2013; Huang & Woolley, 2012) and ERβ receptors (Kumar et al., 2015; Oberlander & Woolley, 2016) which can impact synaptic transmission by modulating G-protein coupled receptors (Sellers et al., 2015). However, activation of the membrane-associated GPER1 is thought to mediate most of the facilitation of synaptic responses in the hippocampus through changes in cAMP (Evans, 2019) and extracellular signal related kinase (ERK) signaling (Kumar et al., 2015; Filardo et al., 2000).

The effects of ovarian hormones on cognitive processes are likely to involve the modulation of synaptic transmission and neuronal excitability in the entorhinal cortex as well as the hippocampus, but there is little known about the impact of ovarian hormones on excitatory and inhibitory synaptic transmission in the entorhinal cortex. Progesterone also modulates memory function (Barros et al., 2015), can rapidly suppress potentiated EPSPs in the CA1 (Edwards et al., 2000), and can increase CA1 spine density within hours (Woolley & McEwen, 1993). Progesterone's metabolite, allopregnanolone, can suppress excitatory transmission by direct allosteric binding to both synaptic and extra-synaptic $GABA_A$ receptors (Wang, 2011; Nin et al., 2011). In the present study we used subdermal capsules to maintain low levels of E2 in ovariectomized rats, and then examined the effects of E2, progesterone, and allopregnanolone on the amplitudes of excitatory postsynaptic field potentials (fEPSP) in the superficial layers of the entorhinal cortex using excitatory postsynaptic field potential recordings (fEPSPs). Application of agonists for estrogen receptors α, β, and GPER1, and an antagonist for GPER1, were used to determine if effects E2 were mediated by ERα, ERβ, and or GPER1.

METHODS

Ovariectomy and estrogen implants

Female Long-Evans rats were obtained on post-natal day (PD) 56 (Charles River, St. Constantine) and pair-housed with *ad libitum* access to standard lab chow and water. Procedures were conducted following guidelines of the Canadian Council on Animal Care and approved by the Concordia University Animal Research Ethics Committee.

On PD63, animals received bilateral ovariectomies, and a subcutaneous capsule was also implanted in the nape of the neck to maintain low circulating levels of E2, to prevent changes in estrogen receptors and synaptic function that could occur following ovariectomy alone (Woolley

and McEwan, 1993; Shima et al., 2002; Rose'Meyer et al., 2003; Gibbs et al., 2014). Rats were anesthetized with 3% isoflurane in O_2, and ovaries were removed via a single 2 cm lumbar incision parallel to the spine and bilateral tears in the abdominal musculature. E2 capsules were made of 1 cm-long Silastic tubing sealed on both ends with silicone (Dow Corning, I.D. 1.47 mm, O.D. 1.96 mm), and contained 8 mg of 5 % cyclodextrin-encapsulated 17β-estradiol (Sigma Aldrich) in cholesterol (Bioshop Canada). This has been reported to result in serum concentration levels of approximately 20 to 30 pg/ml, similar to circulating levels of E2 during the estrus phase, during our recording period (Mannino, et al., 2005; Quinlan et al., 2008). Animals received injections of buprenorphine every 8-12 hours for 72 hours as a post-surgical analgesic (0.05 mg/kg, s.c.).

Slice preparation and recordings

All fEPSP recordings were obtained using slices from ovariectomized female rats at least 7 days following ovariectomy, between PD70 and PD86 (mean PD76 ± 0.43 days; N = 119). Animals were deeply anaesthetized with isoflurane, and brains were rapidly extracted and cooled in oxygenated (95 % O_2 / 5 % CO_2) high sucrose artificial cerebrospinal fluid (ACSF; 4 °C) containing (in mM) 2 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 250 sucrose, 10 dextrose and 0.5 $CaCl_2$. Horizontal, 400 µm-thick sections, were obtained in cold oxygenated ACSF using a vibratome (Leica, VT1200). Sections were collected from approximately -8.1 mm to -6.1 mm ventral to Bregma (Paxinos & Watson, 1998). Slices were stored at 34 ºC for 30 min in normal ACSF containing (in nM), 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose, and then kept for at least 30 min at room temperature (22-24ºC) prior to recordings.

Slices were placed on a nylon net in a gas-fluid interface recording chamber (Fine Science Tools) and all experiments were conducted while slices were kept at room temperature (22-24ºC) and continuously perfused with oxygenated normal ACSF (1.5 - 2.0 ml/min) and exposed to a humidified 95% O_2 / 5% CO_2 atmosphere. Recording electrodes were pulled from borosilicate glass (1.0 mm O.D) using a horizontal puller (Sutter Instruments, P97) and filled with ACSF (5 MΩ). Electrodes were positioned in layer I of the lateral entorhinal cortex with the aid of a dissecting microscope (Leica, MS5) in recording sites just rostral to the most posterior aspect of the slice, at or below the rhinal sulcus (-6.1 to -7.6 mm ventral to Bregma). Bipolar

stimulating electrodes were constructed from two tungsten electrodes (0.8 - 1 MΩ, FHC Inc.) and the tips were placed in the middle of layer I, parallel to the cortical surface. The recording electrode was placed, 0.3 - 0.4 mm posterior to the stimulating electrode, in layer I close to the layer II border, where fEPSPs reflecting activation of superficial layer neurons are maximal (Chapman & Racine, 1997; Hamam et al., 2007). Constant current pulses were delivered using a stimulus generator (WPI, Model A300) and isolation unit (Model A360), and polarity was adjusted to minimize stimulus threshold. fEPSPs were amplified (DC-3kHz, Molecular Devices, Axoclamp 2B) and digitized using pClamp8.2 software (20kHz, Digidata 1322A, Molecular Devices). Stimulation intensities were adjusted to evoke fEPSPs with amplitudes of ~65-75 % of the maximal response (35 to 75 μA).

Pharmacology

Synaptic responses were evoked every 30 sec to establish a stable baseline of at least 10 min, followed by 20 min constant drug-application, and 30 min washout in normal ACSF. Pharmacological agents were dissolved in DMSO, stored frozen, and diluted in ACSF with a final concentration of 0.1 % DMSO. Effects of estrogen receptor activation were assessed by application of 10 nM 17β-estradiol (Sigma Aldrich) (Smejkalova & Woolley, 2010; Huang & Woolley, 2012) similar to concentrations thought to be synthesized locally within the hippocampus (Hojo et al., 2008). Separate recordings also assessed effects of progesterone (100 nM; Tocris) and its metabolite allopregnanolone (1 μM; 5α-pregnan-3a-ol-20-one; Tocris). Control recordings were obtained during application of 0.1 % DMSO alone. Animals used in the study of progesterone or allopregnanolone received a subcutaneous injection of E2 (10 μg in 0.1 ml sesame oil) 12-16 hours prior to obtaining slices. Effects of E2 on progesterone receptors in the entorhinal cortex are not known, but E2 can increase progesterone receptor synthesis and availability in the hippocampus and hypothalamus (Parsons et al., 1980; Guerra-Araiza et al., 2002; Micevych & Sinchak, 2013).

The effects of selective agonists of estrogen receptors ERα, ERβ, or GPER1 were assessed by application of the ERα agonist propylpyrazoletriol (PPT, 100 nM; Tocris Bioscience; Kumar et al., 2015; Oberlander & Woolley, 2016), the ERβ agonist diarylpropionitrile (DPN, 1 μM; Tocris Bioscience; Kumar et al., 2015), or the GPER1 agonist G1 (100 nM, Tocris Bioscience; Kumar et al., 2015; Oberlander & Woolley, 2016). The

contribution of GPER1 receptors to the potentiation of fEPSP amplitude by E2 was also assessed by application of 10 nM E2 for 20 min during constant bath application of the selective GPER1 antagonist, G15 (1 μM; Cayman Chemical; Kumar et al., 2015; Sarchielli et al., 2020).

Paired-pulse tests were used to determine if changes in fEPSP amplitude induced by E2 or G1 could be due to either enhanced presynaptic transmitter release, or enhanced postsynaptic responsiveness (Kumar et al., 2015; Carter & Chapman, 2019). Ten pairs of pulses, with an interpulse interval of 20 ms, were delivered once every 30 sec at the beginning of the baseline period, to reduce possible effects at the onset of drug application, at the end of the drug-application period, and at the end of the washout period.

Data analysis

Peak fEPSPs amplitudes were measured using pClamp 8.2 software (Molecular Devices). Grouped data were plotted by expressing fEPSP amplitudes for each slice as a percentage of the average amplitude of the baseline responses, and each point indicates the mean of the averaged amplitudes of four consecutive responses over a 2 min period, plus or minus one standard error of the mean. Paired-pulse facilitation ratios were expressed as the amplitude of the response to the second pulse divided by the amplitude of the response to the first pulse. The average amplitudes of fEPSPs obtained during the 10-min baseline period, and the last 5 minutes of both the drug-application and washout periods were compared using separate 2-way ANOVAs to assess drug effects, and their reversibility, relative to responses recorded during control application of DMSO. All data are reported as the mean plus or minus one standard error of the mean.

RESULTS

E2, progesterone, and allopregnanolone

Application of E2 (10 nM; Smejkalova & Woolley, 2010; Huang & Woolley, 2012) led to a significant increase in the amplitude of fEPSPs in the lateral entorhinal cortex (Figure 2.1A, B). Field EPSP amplitudes increased to 108 ± 1.3 % of baseline levels after 20-min application of E2 (n=19, PD72 - 83), and remained at 102.1 ± 2.0 % of baseline during application of 1% DMSO (n=17, PD70 - 86). The increase in fEPSP amplitude during E2 application was significantly greater than the change observed in control slices as reflected by the significant interaction of time and drug condition ($F_{1,34} = 6.52$, $p = 0.015$). The increase in fEPSP amplitude

reversed during the 30-min washout period, and there were no significant differences in amplitudes during wash relative to the baseline in slices exposed to E2 (100.4 ± 2.2 of baseline) or DMSO (99.0 ± 3.1 of baseline) ($F_{1,34} = 0.125$, $p = 0.726$). Application of E2 therefore induces a transient increase in evoked synaptic responses in the entorhinal cortex.

The potentiation of fEPSP amplitude induced by E2 could result from increased presynaptic release of glutamate or an increase in postsynaptic responsivity. We therefore examined paired-pulse facilitation ratios, which remain stable when increases in EPSPs are due to postsynaptic mechanisms, and are reduced when EPSPs are facilitated due to increased transmitter release (Kumar et al., 2015; Carter & Chapman, 2019). In comparison to paired-pulse responses recorded at the start of the baseline period, after 20-min application of E2 there was an increase in the amplitude of the response to the first pulse (109.7 ±2.6 % of baseline; $F_{2,36} = 5.53$, $p < 0.01$), but no significant change in paired-pulse ratios, which were 0.588 ± 0.053 during baseline and 0.559 ± 0.048 in E2 ($n = 19$, $F_{2,36} = 1.94$, $p = 0.16$) (Figure 2.1C). The increase in fEPSP amplitude is therefore likely due to postsynaptic changes rather than to an increase in transmitter release.

Progesterone and its metabolite, allopregnanolone, can modulate glutamatergic synaptic transmission in the hippocampus (Edwards et al., 2000; Wang, 2011), and we therefore tested their effects on fEPSP amplitude in the entorhinal cortex (Figure 2.2). In control slices, fEPSP amplitude was 100.5 ± 3.1 % of baseline at the end of DMSO application, and 94.7 ± 4.0 of baseline at the end of the washout period (n=9, PD70 - 86). Application of 100 nM progesterone (n = 11, PD70 - 86) did not result in a significant change in fEPSP amplitude (103.0 ± 2.5 % of baseline) relative to control slices ($F_{1,18} = 0.43$, $p = 0.523$) and there was also no significant difference in responses at the end of the washout period (96.6 ± 3.4 % of baseline) relative to control slices ($F_{1,18} = 0.13$, $p = 0.72$). Application of 1 µM allopregnanolone (n=7, PD70 - 83) did not result in a significant change in fEPSP amplitude relative to control slices (101.7 ± 1.8 % of baseline; $F_{1,14} = 0.10$, $p = 0.761$), nor a significant differences in responses at the end of the washout period (101.3 ± 1.9 % of baseline; $F_{1,14} = 1.82$, $p = 0.20$). Therfore, neither application of progesterone nor allopregnanolone for 20 minutes induced marked changes in excitatory synaptic transmission in the entorhinal cortex.

Estrogen receptors

The contributions of estrogen receptors ERα and ERβ to the E2-induced potentiation of fEPSP amplitude was assessed using the specific ERα receptor agonist PPT, and ERβ receptor agonist DPN. Application of the ERα receptor agonist, PPT (100 μM) for 20 min did not significantly affect fEPSP amplitude (104 ± 3.0 % of baseline; n = 15, PD70 - 79; $F_{1,30} = 0.32$, p = 0.579), and there was also no significant change relative to control slices at the end of the washout period (98.0 ± 3.3 % of baseline; $F_{1,30} = 0.05$, p = 0.825; Figure 2.3A). This suggests that ERα receptors do not contribute substantially to the potentiation of fEPSP amplitude induced by E2. Similarly, the ERβ receptor agonist, DPN (1 μM) did not significantly affect fEPSP amplitude (102 ± 2.0 % of baseline; n=14, PD70 - 86; $F_{1,29} = 0.012$, p = 0.914), and there was no significant change in fEPSP amplitude at the end of the washout period (102.6 ± 2.8 % of baseline; $F_{1,29} = 0.68$, p = 0.416) (Figure 2.3B). Neither ERα nor ERβ estrogen receptors are therefore likely to contribute to the potentiation of fEPSP amplitude induced by E2.

Activation of GPER1 receptors can have rapid and transient effects on synaptic transmission (Kumar et al., 2015; Lebesgue et al., 2010), and we therefore assessed whether the specific GPER1 receptor agonist G1 could enhance fEPSP amplitude in the entorhinal cortex. Application of 100 nM G1 for 20 min significantly increased fEPSP amplitude (n = 17, PD70 - 81; $F_{1,16} = 9.02$, p = 0.008), and the size of the increase (110.3 ± 3.4 % of baseline; Figure 2.4A) was similar to that induced by E2 (108.0 ± 1.3 %; Figure 2.1A). The increase in fEPSP amplitude was transient, and reversed at the end of the washout period (99.3 ± 3.0 % of baseline; $F_{1,16} = 0.05$, p = 0.826). Similar to results for application of E2, G1 resulted in no significant change in paired-pulse ratio which was 0.693 ± 0.036 during baseline and 0.738 ± 0.069 in G1 (n = 16; $F_{1,15} = 0.90$, p = 0.36; not shown).

To further determine if the potentiation of fEPSP amplitude induced by E2 is mediated by activation of GPER1 receptors, slices were exposed to constant bath application of the GPER1 antagonist, G15 (1 μM; Kumar et al., 2015; Sarchielli et al., 2020) for 10 minutes, followed by co-application of 10 nM E2 for 20 min (Figure 2.4B). The presence of G15 blocked the potentiation of fEPSP amplitude induced by E2, and fEPSP amplitude remained at 98.6 ± 2.5 % of baseline following 20-min E2 application (n= 11; PD70 - 86). There was no significant difference between fEPSP amplitude recorded during the baseline period and after application of E2 ($F_{1,10} = 0.30$, p = 0.595), but after the 30 min washout of E2 in the presence of G15, fEPSP

amplitude was significantly reduced relative to the baseline period (92.9 ± 2.4 % of baseline; $F_{1,10}$ =11.41, p = 0.007). Comparison with slices that received E2 alone (see Figure 2.1A) showed that G15 significantly blocked the effects of E2 ($F_{1,28}$ = 13.29, p = 0.001), indicating that acivation of GPER1 receptors is necessary for the increase in fEPSP amplitude induced by E2.

DISCUSSION

The entorhinal cortex is tightly interconnected with both the neocortex and hippocampal formation, and the effects of estrogens and progesterone on synaptic transmission in the entorhinal cortex are likely to contribute to cognitive processes through multiple intracellular cellular signaling mechanisms (Witter et al., 2017; Burwell, 2000; Finney et al., 2020). Estrogens and progesterone have genomic effects on synaptic function that are expressed over hours to days (Rossetti et al., 2016; Kim et al., 2016; Woolley & McEwen, 1993). In addition, membrane-associated estrogen receptors can induce rapid effects on synaptic function through multiple mechanisms involving ERα (Huang & Woolley, 2012), ERβ (Kramár et al. 2009; Zang et al., 2020), and GPER1 receptors (Lebesgue et al., 2010; Kumar et al., 2015). Here, in slices from ovariectomized rats that received subdermal implants to maintain E2 at levels similar to estrus (Quinlan et al., 2008), we found that 20-min application of 10 nM E2 resulted in an increase in the amplitude of field EPSPs in the entorhinal cortex in vitro that was reversed following washout in normal ACSF. The concentration used here is substantially higher than circulating estrogen levels, but similar to the concentration range resulting from locally synthesized estrogen that has been observed in the hippocampus (Hojo et al., 2008). This increase is consistent with reports in the CA1-CA3 regions of the hippocampus which show similar increases in both field and intracellular EPSPs after application of E2 in vitro (Sharrow et al., 2002; Rudick & Woolley, 2003; Kramár et al., 2009; Smejkalova & Woolley, 2010). This potentiation of excitatory synaptic responses in the superficial layers of the entorhinal cortex may enhance the salience of cortical sensory and associational inputs, and may also enhance functions of the hippocampal formation by combining synergistically with the estrogenic potentiation of synaptic transmission in the targets of entorhinal neurons in the hippocampal subfields (Kim et al., 2006).

The potentiation of synaptic responses induced by E2 in the entorhinal cortex was mediated by activation of GPER1 receptors, but ERα or ERβ receptors do not appear to contribute. The synaptic potentiation induced by E2 was not mimicked by the ERα or ERβ

receptor agonists, PPT nor DPN. However, the GPER1 receptor agonist G1 potentiated fEPSPs, and the potentiation induced by E2 was blocked by the selective GPER1 receptor antagonist, G15. The significant reduction in fEPSP amplitude after washout of E2 during continuous application of G15 could suggest that ongoing activation of GPER1 receptors may help maintain excitatory synapses (Gonzalez de Valdivia et al., 2017), but this is highly speculative because modest reductions in fEPSPs were also observed late in the recording period in other groups (Figure 2.2). Application of the ERα agonist PPT was associated with a non-significant increase of about 4 % in fEPSP amplitude, but there is only minimal expression of ERα in the entorhinal cortex (Kritzer et al., 2002) and this trend may be due to non-selective activation of GPER1 receptors by PPT (Petrie et al., 2013) rather than to activation of ERα (Tanaka & Sokabe, 2013; Huang & Woolley, 2012). The entorhinal cortex shows strong expression of ERβ (Kritzer et al., 2002; Merchenthaler et al., 2004; Shima et al., 2003) with intense expression in parvalbumin and calbindin containing interneurons in layers III to VI (Kritzer et al., 2002; Blurton-Jones & Tuszynski, 2002, 2006). The rapid potentiation of synaptic responses mediated by ERβ in the hippocampus has been linked to both increased glutamate release and postsynaptic cytoskeletal changes (Smejkalova & Woolley, 2010; Kramár et al. 2009). However, within the superficial layers of the entorhinal cortex, the rapid effects of estrogens appear to be due to activation of GPER1 receptors, and this is consistent with other findings in the hippocampus where GPER1 receptors mediate the synaptic potentiation induced by E2 (Kumar et al. 2015; Lebesgue et al., 2010; Oberlander & Woolley, 2016). Slices were obtained in ovariectomized animals that received subdermal E2 implants, to maintain E2 at levels similar to estrus (Mannino, et al., 2005; Quinlan et al., 2008) and to avoid changes in estrogen receptors and synaptic function that can be induced by ovariectomy alone (Woolley & McEwen, 1993; Shima et al., 2002; Rose'Meyer et al., 2003). Kumar et al. (2015), however, have reported a rapid GPER1-mediated increase in synaptic function following ovariectomy without E2 replacement, suggesting that E2 implants may not have been required to support the GPER1-mediated potentiation observed here.

GPER1 signaling mechanisms

GPER1 receptors are localized to the cell membrane and endoplasmic reticulum and can modulate neuronal function through several signaling pathways (Alexander et al., 2017; Lu & Herndon, 2017). We found that application of E2 or the GPER1 agonist G1 potentiated fEPSP

amplitude without affecting paired-pulse ratios, suggesting that the potentiation was due to an increase in the postsynaptic response rather than increased transmitter release (Hamam et al., 2007). Kumar et al. (2015) also report the potentiation of synaptic responses in the hippocampus via activation of GPER1 with no change in paired-pulse ratio. The increase in fEPSP amplitude could result from postsynaptic effects of GPER1 on glutamate receptors, but the GPER1 could also facilitate fEPSPs indirectly without affecting the paired-pulse ratio by suppressing inhibitory transmission (Mukherjee et al., 2017; Murphy et al., 1998; Tabatadze et al., 2015). Estrogen can facilitate EPSPs by disinhibiting hippocampal neurons, and the disinhibition has been associated with increased dendritic spines in the hippocampus in vivo (Rudick Woolley 2001; Rudick et al., 2003). The effects of GPER1 activation on inhibitory synaptic transmission in the entorhinal cortex are not known, but it has been found to increase inhibitory transmission in the amygdala (Tian et al., 2013). Thus, the potentiation of fEPSP amplitude observed here may be due to a GPER1-mediated reduction in synaptic inhibition in the entorhinal cortex, which could be expressed without change in paired-pulse ratio. The rapid potentiation of fEPSP amplitude in the entorhinal cortex induced by E2 may be initiated by activation of the G-protein α-subunit by GPER1 and resulting modulation of adenylyl cyclase and cAMP signaling (Alexander 2017; Lu & Herndon, 2017; Finney et al, 2020). Activation of GPER1 can also lead to activation of epidermal growth factor receptor (EGFR) and stimulation of extracellular signal regulated kinase (ERK). ERK signaling has been shown to mediate increased EPSPs mediated by activation of GPER1 (Kumar et al., 2015).

Increases in intracellular calcium may contribute to the potentiation of synaptic responses induced by GPER1 activation. Activation of GPER1 can increase the amplitude of glutamate-induced calcium transients in dendritic spines (Oberlander & Woolley, 2016). Similarly, activation of GPER1 on both the cell membrane and the endoplasmic reticulum can cause release of calcium ions from internal stores through phospholipase C and IP_3 signaling (Brailoiu et al., 2007; Alexander, 2017), and increases in intracellular calcium can modulate AMPA receptor phosphorylation and trafficking (Zadran et al., 2009). In addition, GPER1 may influence synaptic function by modulating the activation of other G protein-coupled receptors. Akama et al. (2013), reported that membrane bound GPER1 receptors were often colocalized with the scaffolding protein PSD-95 and can dimerize with 5-HT1A, corticotropin, and progestin receptors, and they speculated that GPER1 may also influence the activity of other G protein-

coupled receptors as well. It has also been suggested that GPER1 may affect AMPA receptor dynmics through interaction with PSD-95 and SAP97 (Nair et al, 2013; Waters et al., 2015; Oberlander & Woolley, 2016).

Progesterone

Progesterone can enhance hippocampus-dependent cognitive processes through genomic and non-genomic effects on synaptic function in the hippocampus (Frye et al., 2009; Prakapenka et al. 2018; Frye & Walf, 2008). Progesterone receptors are expressed throughout the brain (Meffre et al., 2013), but the entorhinal cortex shows little expression of progesterone receptor mRNA (Kato et al., 1994). Administration of E2 can promote the synthesis and expression of progesterone receptors in the hippocampus and hypothalamus (Parsons et al., 1980; Guerra-Araiza et al., 2002; Alves et al., 2000; Micevych & Sinchak, 2013). However, although we conducted recordings in slices obtained from animals that received a prior bolus injection of E2, we found no effect of 20-min application of progesterone on synaptic responses in the entorhinal cortex. Allopregnanolone is a major metabolite of progesterone that can inhibit excitatory transmission by directly potentiating activation of $GABA_A$ receptors (Wang, 2011; Schverer et al., 2018; Nin et al., 2011). In the hippocampus, allopregnanolone application for 30 min results in a reduction of excitatory synaptic transmission (Edwards et al., 2000). Variations in inhibition affect excitatory synaptic responses in the entorhinal cortex (Ferreira et al., 1992), and we expected allopregnanolone to suppress fEPSP amplitude, but we found that fEPSP amplitude was unaffected. This may be due to regional differences in $GABA_A$ subunit composition (Wang, 2000), and intracellular recordings may be needed to better assess the effects of allopregnanolone on inhibitory synaptic responses in entorhinal neurons.

Functional significance

Variations in the level of ovarian hormones are thought to modulate cognitive functions by affecting excitatory synaptic transmission and neuronal excitability in multiple brain regions. A major focus of the effects of estrogens on cognition has been on the powerful effects of estrogens on hippocampal function that involve both genomic, and more rapid non-genomic effects, on inhibitory and excitatory synaptic transmission. The present results indicate that E2 can rapidly potentiate excitatory synaptic inputs to the superficial layers of the lateral entorhinal cortex, and this is likely to make an important contribution to the mechanisms mediating the

effects of estrogens on cognitive function. Variations in estrogen may affect both cortical inputs to the hippocampus (Kerr et al., 2007) as well as the use of cognitive strategies involving the lateral entorhinal cortex which may depend on object identification memory and associations with spatial location (Deshmukh & Knierim, 2011; Knierim et al., 2013; Van Cauter et al., 2013). Although the findings presented here demonstrate a role for GPER1 receptors in modulating synaptic function in the entorhinal cortex in the female rat, the potential role of these receptors in synaptic function in males may well differ (Oberlander & Woolley, 2016) and has yet to be determined.

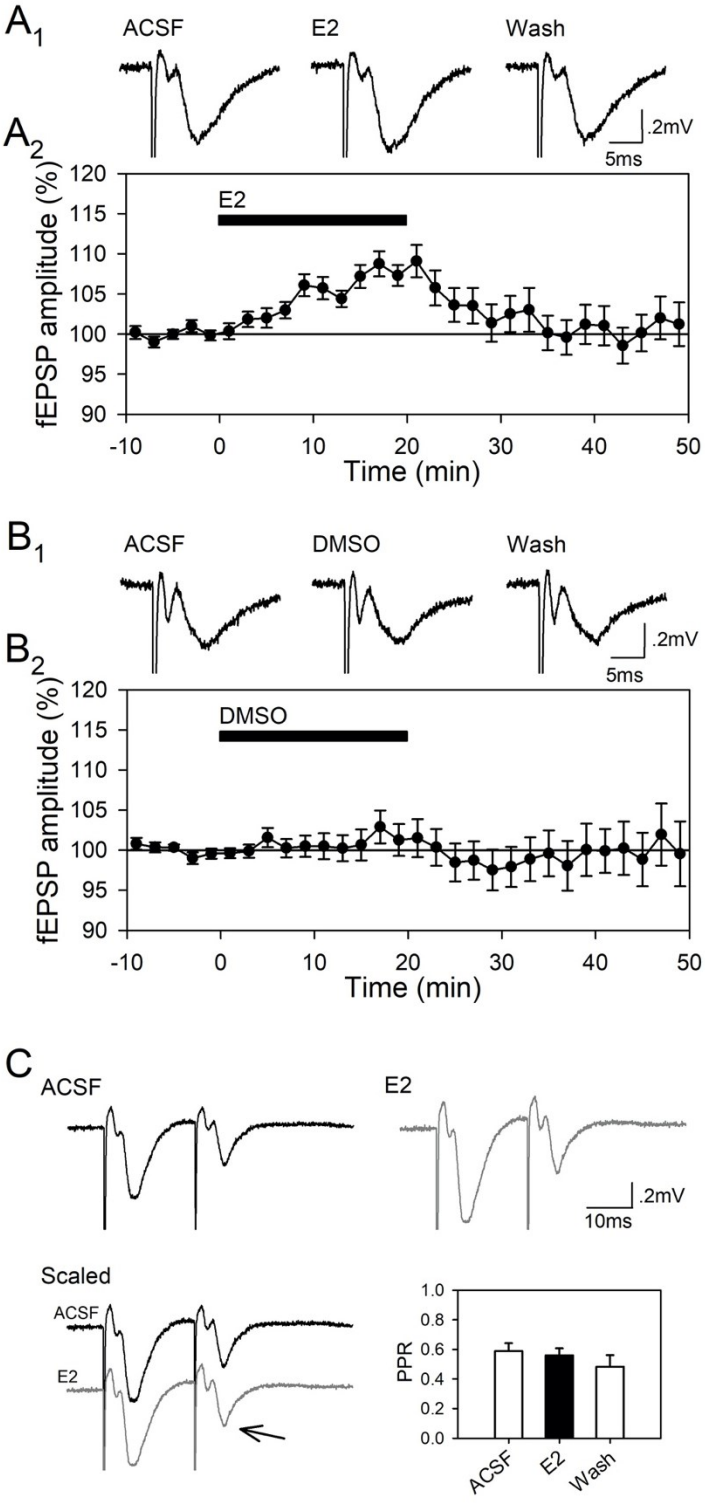
A1
ACSF
E2
Wash
.2mV
5ms
A2
fEPSP amplitude (%)
E2
Time (min)
B1
ACSF
DMSO
Wash
.2mV
5ms
B2
fEPSP amplitude (%)
DMSO
Time (min)
C
ACSF
E2
.2mV
10ms
Scaled
ACSF
E2
PPR
ACSF
E2
Wash

Figure 2.1. Bath-application of 17-β estradiol (E2) results in a reversible increase in the amplitude of evoked field excitatory postsynaptic potentials (fEPSPs) in the lateral entorhinal cortex in vitro. **A**. The amplitudes of fEPSPs evoked by layer I stimulation increased significantly during 20-minute bath application of 10 nM E2 (black bar) and returned to baseline following a 30-minute washout period. Representative fEPSP traces show averages of five consecutive responses obtained at the end of each recording period (A_1), and mean amplitudes for each time point (± one SEM) are shown for the group of slices tested (n = 19; A_2). The same conventions apply in subsequent panels and Figures. **B**. Application of the vehicle DMSO (0.1%) did not significantly affect field EPSP amplitudes (n = 17). **C**. Paired-pulse recordings obtained during the baseline period in ACSF (top left) versus during application of E2 (top right) were compared to assess if the increase in fEPSP amplitude may be due to changes in pre- versus postsynaptic mechanisms. Traces have been scaled to the amplitude of the first response in each pair and offset vertically (bottom left) to highlight the similarity in the scaled amplitudes of the second responses in each pair (arrow indicates the second response recorded in E2). There was no significant change in paired-pulse ratio (PPR).

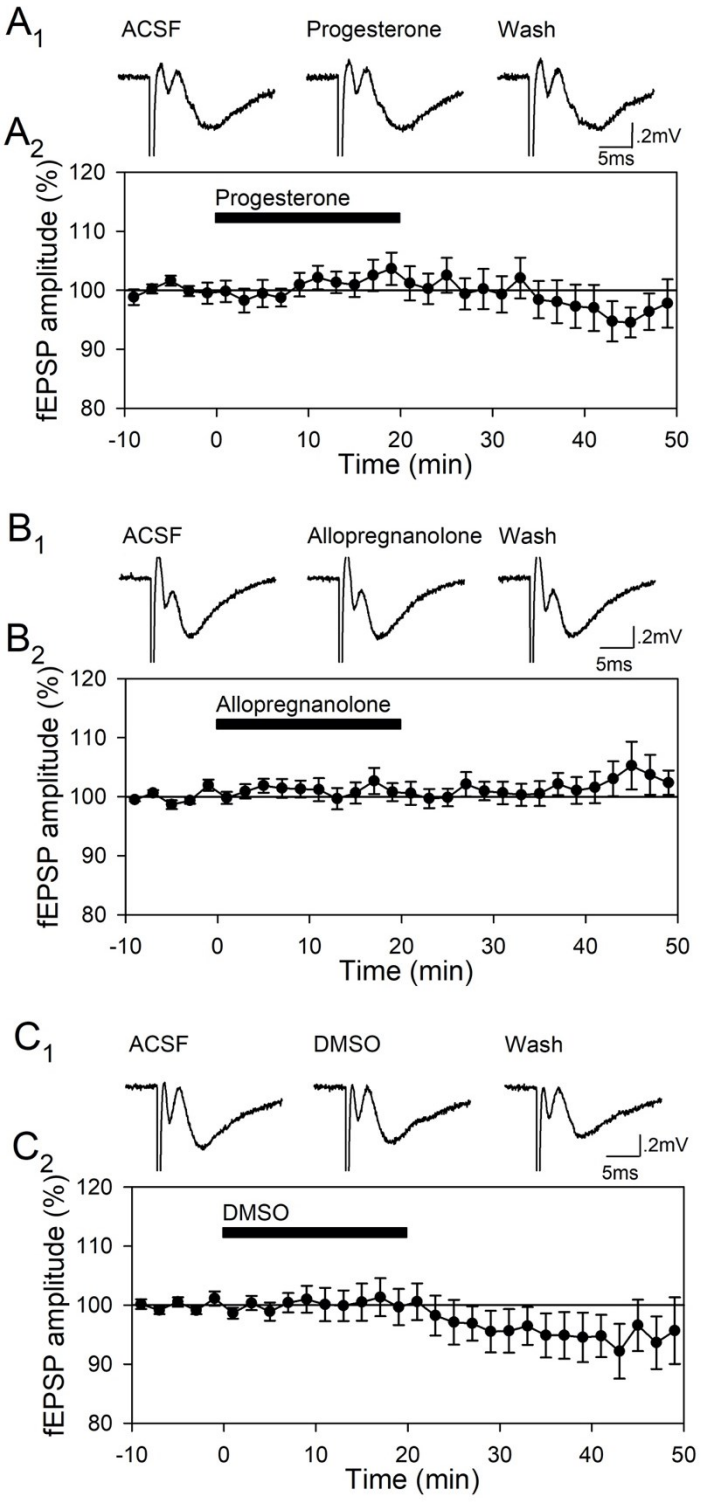
A1
ACSF
Progesterone
Wash
.2mV
5ms
A2
fEPSP amplitude (%)
Progesterone
Time (min)
B1
ACSF
Allopregnanolone
Wash
.2mV
5ms
B2
fEPSP amplitude (%)
Allopregnanolone
Time (min)
C1
ACSF
DMSO
Wash
.2mV
5ms
C2
fEPSP amplitude (%)
DMSO
Time (min)

Figure 2.2. Neither application of progesterone nor allopregnanolone significantly altered the amplitude of field excitatory postsynaptic potentials (fEPSP) in the entorhinal cortex relative to control slices treated with DMSO. **A**. Representative averaged fEPSPs (A_1) are shown before, during and after 20-min application of 100 nM progesterone, and the mean fEPSP amplitude was stable in the group of slices tested (n = 10; A_2). **B**. Application of 1µM allopregnanolone also had no significant effect on fEPSP amplitude (n = 7). **C**. Representative responses and mean fEPSP amplitudes are shown for the group of control slices exposed to the drug vehicle DMSO (0.1%; n = 9).

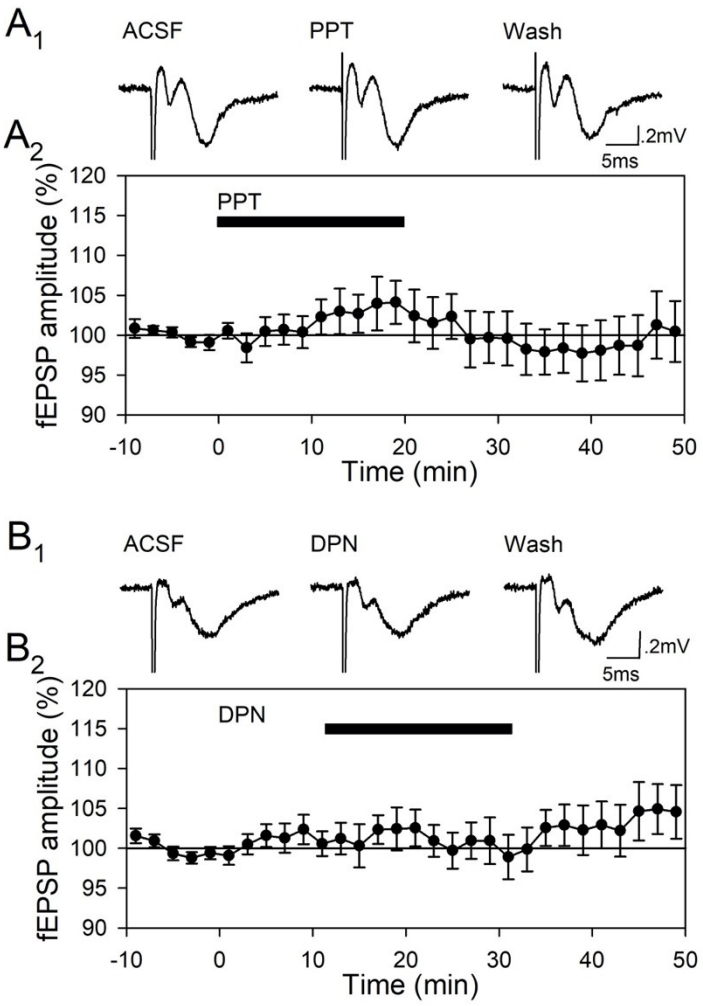
A1
ACSF
PPT
Wash
.2mV
5ms
A2
PPT
fEPSP amplitude (%)
120
115
110
105
100
95
90
-10
0
10
20
30
40
50
Time (min)
B1
ACSF
DPN
Wash
.2mV
5ms
B2
DPN
fEPSP amplitude (%)
120
115
110
105
100
95
90
-10
0
10
20
30
40
50
Time (min)

Figure 2.3. Neither the estrogen receptor α agonist PPT, nor the estrogen receptor β agonist DPN, resulted in a significant increase in fEPSP amplitude. **A**. Application of the ERα agonist PPT (100 nM) for 20 min was associated with small increases in fEPSPs in several slices (A_1) but the effect was not significant in the group of slices tested (n = 15; A_2). **B**. Application of the ER β agonist DPN (1 μM) for 20 min did not significantly affect representative traces (n = 14; B_1) or averaged fEPSP amplitude in the group of slices tested (B_2).

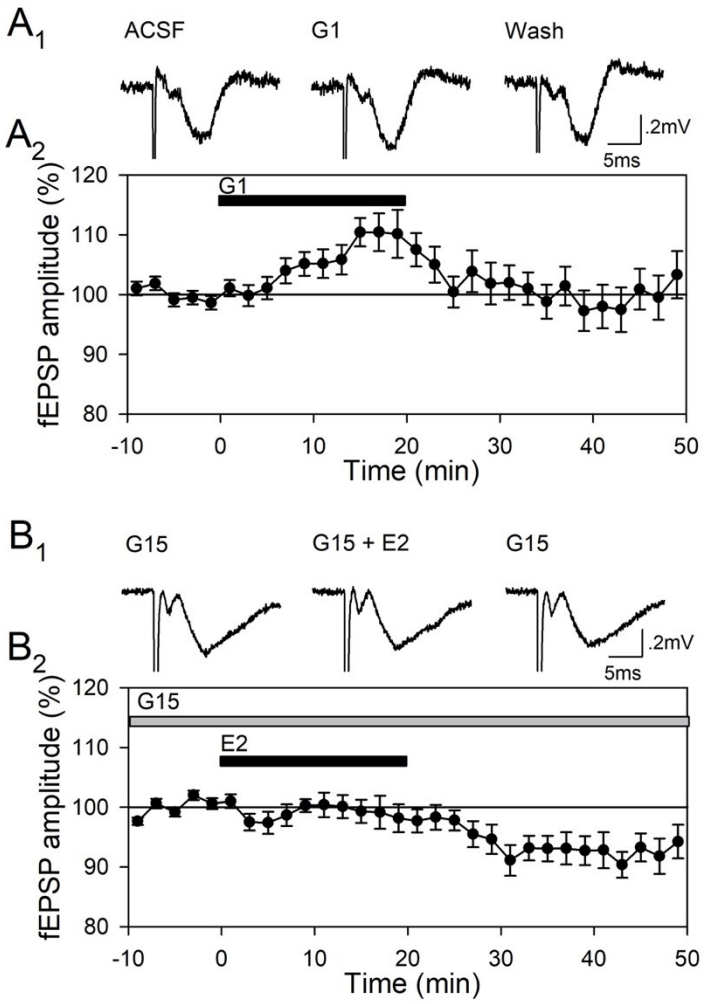
A1
ACSF
G1
Wash
.2mV
5ms
A2
G1
fEPSP amplitude (%)
120
110
100
90
80
-10
0
10
20
30
40
50
Time (min)
B1
G15
G15 + E2
G15
.2mV
5ms
B2
G15
E2
fEPSP amplitude (%)
120
110
100
90
80
-10
0
10
20
30
40
50
Time (min)

Figure 2.4. GPER1 receptor activation is required for the enhancement of fEPSP induced by application of E2. **A**. Application of the specific GPER1 agonist G1 (100 nM) resulted in a significant increase in fEPSP amplitude similar to that induced by E2. Representative fEPSP traces show averages of five consecutive responses obtained at the end of each recording period (A_1), and mean amplitudes for the group of slices tested are shown (n = 17; A_2). **B** Constant bath application of the GPER1 antagonist G15 (1 μM; grey bar), prevented the increase in fEPSP amplitude induced by application of E2 (n = 11).

CHAPTER 3

17β-ESTRADIOL REDUCES $GABA_A$ AND NMDA RECEPTOR-MEDIATED SYNAPTIC CURRENTS IN THE LATERAL ENTORHINAL CORTEX

ABSTRACT

Estrogens are believed to contribute to cognitive function through the modulation of synaptic transmission in the cortex and hippocampus. Administration of 17β-estradiol (E2) can increase dendritic spine densities and enhance excitatory synaptic transmission in the hippocampus. Previous work has shown that estrogens can facilitate excitatory synaptic transmission in the entorhinal cortex via activation of the G protein-coupled estrogen receptor-1 (GPER1). However, the way GPER1 activation modulates synaptic processes to facilitate synaptic transmission is unclear. The present study assessed the effects of acute E2 administration (10 nM) on excitatory (AMPA and NMDA) and inhibitory ($GABA_A$) receptor-mediated currents in layer II/III neurons in slices of rat entorhinal cortex. Female Long-Evans rats were ovariectomized on postnatal day (PD) 63 and implanted with a subdermal E2 capsule to maintain continuous levels of E2 on par with metestrus. Electrophysiological recordings were obtained from brain slices containing the entorhinal cortex between PD70 and PD86. Pharmacologically isolated excitatory and inhibitory postsynaptic currents were recorded from principal neurons in layers II and III of the lateral entorhinal cortex. Application of E2 for 20 min did not significantly change AMPA receptor-mediated excitatory currents from baseline. However, E2 reduced NMDA receptor-mediated excitatory currents, and this effect persisted following removal of E2 during the 20-min wash off period. $GABA_A$ receptor-mediated inhibitory currents were markedly reduced during application of E2, and returned towards baseline levels during the 20-min washout period. These results suggest that strong reductions in GABAergic inhibition in the entorhinal cortex may be a mechanism through which E2 increases excitatory synaptic transmission in the entorhinal cortex.

Cognitive processes in females are influenced by fluctuations in estrogens associated with the reproductive cycle. Fluctuations in the concentrations of estrogens have been associated with shifts in the preferred use of navigational strategies on spatial memory tasks in humans (Holden, 2010; Hussain et al., 2016), and improvements to attention and emotional memory recall when estrogens are elevated (Sommer et al., 2018). Female rats demonstrate similar shifts in navigational strategy depending on the phase of estrous and the concentration of estrogens present (Korol & Kolo, 2002). These fluctuations can also affect rats' performance on object memory (Van Goethem et al., 2012), social learning (Ervin, Mulvale, et al., 2015), and fear conditioning tasks (Day et al., 2005). Disruption of circulating estrogens during the perimenopausal transition or following surgical menopause can impair performance on these tasks. Administration of supplementary estrogens has been found to prevent or reduce impairment in some studies (Engler-Chiurazzi et al., 2017; Newhouse & Dumas, 2015).

Estrogens are thought to exert their influence via the activation of estrogen receptors (ER) α, β, and the G protein-coupled estrogen receptor-1 (GPER1) that are expressed in regions throughout the brain. In general, regions that express estrogen receptors have a high density of GPER1 receptors, with varying degrees of ERα and ERβ expression (Hazell et al., 2009). Activation of estrogen receptors can induce delayed changes in gene expression and rapid modulation of synaptic function. In the hippocampus, administration of 17β-estradiol (E2) can rapidly increase dendritic spine densities (Woolley & McEwen, 1992), modulate $GABA_A$ receptor trafficking (Mukherjee et al., 2017), and enhance excitatory synaptic transmission (Kumar et al., 2015). Rapid enhancements of excitatory transmission have been linked to the activation of GPER1 receptors (Batallán Burrowes et al., 2021; Kumar et al., 2015; Oberlander & Woolley, 2016), but activation of both ERα and ERβ can also contribute (Kumar et al., 2015; Oberlander & Woolley, 2016).

The entorhinal cortex is thought to promote cognitive function by providing the hippocampus with much of its sensory and associational inputs (Knierim et al., 2013; Nilssen et al., 2019). The entorhinal cortex can be divided into the medial entorhinal cortex and the lateral entorhinal cortex. The medial entorhinal cortex is believed to contribute to spatial mapping and navigation, while the lateral entorhinal cortex is associated with olfaction, object memory, and memory of object location (Knierim et al., 2013). Both regions receive projections from multiple

associational cortices, and send projections to the hippocampal formation in a region-specific manner. Entorhinal cortex neurons express estrogen receptors, with a high density of GPER1 receptor expression throughout the medial and lateral entorhinal cortex (Hazell et al., 2009). ERβ and ERα expression have also been observed in the entorhinal cortex to a lesser degree, and in distinct layers. ERβ expression is localized to layers III-VI of the entorhinal cortex, while ERα is more sparsely expressed in layers V and VI (Hazell et al., 2009; Kritzer, 2002; Merchenthaler et al., 2004).

Despite the contributions of the entorhinal cortex to cognition and its close connections with the hippocampus, there is limited research investigating the effects of estrogens on synaptic function and behavior in the entorhinal cortex. Previous work from our lab has demonstrated that E2 can facilitate excitatory synaptic transmission in the entorhinal cortex *in vitro* via activation of GPER1 receptors (Batallán Burrowes et al., 2021). Application of E2 for 20 min induced a reversable increase in the amplitude of electrically evoked excitatory post-synaptic field potentials (fEPSPs) recorded in the lateral entorhinal cortex of slices from ovariectomized rats with low levels of circulating E2. The effect of E2 was mimicked by the GPER1 agonist G1, and blocked by the GPER1 antagonist G15, but ERα and ERβ agonists had not effect (Batallán Burrowes et al., 2021). The facilitation of fEPSPs induced by E2 could result from an increase in excitatory transmission mediated by AMPA or NMDA glutamate receptors, but it could also result from a reduction in inhibitory $GABA_A$ receptor-mediated inhibition Brief application of E2 has been found to enhance AMPA receptor responses via activation of GPER1 in the hippocampus (Oberlander & Woolley, 2016), and E2 can also enhance NMDA receptor-mediated currents (Foy et al., 1999) and NMDA receptor-mediated calcium transients (Pozzo-Miller et al., 1999) in hippocampal neurons. Additional work by Kramár et al. (2009) found that E2 facilitated isolated AMPA responses in hippocampal CA1 neurons, but did not affect either NMDA responses or inhibitory synaptic responses. Increases in spine density induced by E2 in the hippocampus have also been found to be mediated in part by a reduction in synaptic inhibition that results in increased excitability (Murphy et al., 1998; Tabatadze et al., 2015). This is supported by findings that E2 can rapidly reduce GABAergic inhibition in cultured neurons via relocation of GABA receptors away from synapses (Mukherjee et al., 2017).

The present study used *in vitro* whole-cell voltage clamp recordings from layer II and III lateral entorhinal cortex neurons to assess the effects of E2 on pharmacologically isolated AMPA

receptor-mediated excitatory post-synaptic currents (EPSCs), NMDA receptor mediated EPSCs, and $GABA_A$ receptor-mediated inhibitory post-synaptic currents (IPSCs). Brain slices containing sections of the lateral entorhinal cortex were obtained from ovariectomized rats implanted with subdermal E2 capsules to maintain constant low levels of E2 similar to metestrus (Mannino et al., 2005; Almey et al., 2013). Determining the excitatory and inhibitory neurotransmitter receptors that are modulated by E2 in the entorhinal cortex could provide fundamental information regarding the cellular mechanisms through which E2 may promote cognitive function by modulating excitatory transmission in the entorhinal cortex.

METHODS

Ovariectomy and estrogen implants

Female Long-Evans rats were obtained from Charles River (St. Constantine, Canada) at post-natal day (PD) 56. Rats were pair-housed with *ad libitum* access to standard rat chow and water. All procedures were submitted and approved by the Concordia University Animal Research Ethics Committee.

All rats received bilateral ovariectomy on PD 63 and were implanted with a subcutaneous estrogen capsule in the nape of the neck to maintain low levels of E2 on par with levels recorded in metestrus (Mannino et al., 2005). Implants were used to prevent changes to estrogen receptor expression and synaptic function that could occur following ovariectomy alone (Gibbs et al., 2014; Rose'Meyer et al., 2003; Shima et al., 2002; Woolley & McEwen, 1993). Rats were anesthetized with 3% isoflurane in O_2, and ovaries were removed via a single 2 cm lumbar incision parallel to the spine and bilateral tears in the abdominal musculature. Capsules were constructed of 1 cm long Silastic™ tubing (Dow Corning, I.D. 1.47 mm, O.D. 1.96 mm) sealed with silicone on both ends. The capsules contained 8 mg mixture of 5% water-soluble 17β-estradiol (Sigma-Aldrich) in cholesterol (Bioshop Canada; Mannino et al., 2005; Quinlan et al., 2008). To provide analgesia following surgery, animals received buprenorphine (0.05 mg/kg, s.c.) injections every 8 to 12 hours for 72 hours.

Slice preparation

All electrophysiological recordings were obtained from brain slices between 7 and 28 days following ovariectomy (PD 70 to 98). Animals were anaesthetized with isoflurane and decapitated using a guillotine. Brains were rapidly extracted from the skull and cooled in

oxygenated (95% O_2 / 5% CO_2) high sucrose artificial cerebrospinal fluid (ACSF; 4 °C) containing (in mM) 2 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 250 sucrose, 10 dextrose and 0.5 $CaCl_2$. Brain slices (300 µm-thick) were prepared in cold oxygenated ACSF using a vibratome (Leica, VT1200). Slices were stored at 34 ºC for 30 min in normal ACSF containing (in mM), 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$ and 10 dextrose, and then at 21-23 ºC for at least 30 min prior to recordings. To obtain recordings, slices were perfused in 21-23 ºC ASCF at a rate of 2 ml/min under the objective (40X) of an upright microscope (Leica, DM-LFS) equipped with differential interference contrast optics.

Electrophysiological recordings

Recording electrodes for whole-cell recordings were prepared from borosilicate glass pipettes (1.0 mm OD, 3 to 6 MΩ) using a Brown-Flaming pipette puller (Sutter Instruments, P97). Electrodes were filled with different solutions to obtain recordings of AMPA EPSCs, NMDA EPSCs or $GABA_A$ IPSCs. Electrodes used to record AMPA EPSCs were filled with a solution containing (in mM) 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.2-7.3 with KOH). The recording solution for NMDA EPSCs contained 130 cesium methane sulfonate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 10 phosphocreatine di tris, 5 QX314, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.2-7.3 with CsOH). The recording solution for $GABA_A$ IPSCs contained 135 KCl, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 10 phosphocreatine di tris, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.2-7.3 with KOH).

Tight seals were formed (1-3MΩ) on the soma of a layer II or layer III neurons prior to obtaining whole-cell configuration. Cells were allowed to stabilize for 10 to 15 min prior to recordings to allow intracellular recording solutions to diffuse within the neuron and equilibrate with the cytosol. Synaptic responses were evoked using a bipolar stimulating electrode made from two tungsten electrodes (~1 MΩ, FHC Inc.). Electrodes were placed with at least one tip in layer I of the entorhinal cortex about 0.3 mm from the recording electrode. Synaptic responses were evoked by 0.1 ms-duration constant current pulses using a stimulus generator and isolation unit (WPI, Models A300 and A360). Liquid junction potentials that result from differences in ion concentrations between the ACSF and recording solutions were corrected for in the membrane potential values that are reported for AMPA (15 mV), NMDA (9 mV), and GABA (3 mV).

Current and voltage clamp recordings were obtained using an Axopatch 200B amplifier with a low pass filter at 10 kHz, and the recordings were digitized at 20 kHz using an analog-digital converter (Axon Instruments, Digidata 1322A). Recordings were constantly monitored using a digital oscilloscope. The intensity of stimulation pulses was adjusted to evoke synaptic responses approximately 70% of the maximal response.

After obtaining whole-cell configuration, the electrophysiological characteristics of neurons were assessed in current clamp mode by recording membrane potential responses to 500 ms current-step injections at 25 pA intervals (range -200 to +150 pA). Series resistance was estimated by compensating for the discontinuity in voltage response to a -200 pA current pulse, and recordings were accepted if series resistance was less than 25 MΩ. Series resistance was not compensated for during voltage-clamp recordings. Series and input resistance were monitored using a -5 mV step after each evoked response.

Synaptic current recordings were collected in voltage-clamp, at least 10 min after obtaining a whole-cell configuration to allow neurons to acclimate to the intracellular solutions in the recording electrode. All electrically evoked EPSCs and IPSCs were collected in sets of 10, with a 15-sec interval between evoked responses, and a 10-min interval between sets of responses. After recording 2 sets of stable baseline responses, 17β-estradiol (E2; 10 nM; Sigma Aldrich) was applied for 20-min, with sets of evoked responses recorded at 10 and 20 min. Two sets of responses were recorded during the washout of E2 at 10 and 20 minutes after 20-min of E2.

AMPA receptor-mediated EPSCs were obtained at a holding membrane potential of -85 mV during constant bath application of the NMDA blocker (2R)-amino-5-phosphonopentanoate (AP5, 50 μM) and the chloride channel blocker picrotoxin (50 μM). NMDA receptor-mediated EPSCs were recorded at +45 mV in the presence of the AMPA receptor blocker 7-nitro-2,3-dioxo-1,4- dihydroquinoxaline-6-carbonitrile (CNQX, 20 μM), and picrotoxin. $GABA_A$ receptor-mediated IPSCs were recorded at -83 mV in the presence of the AP5 and CNQX. Drugs were stored as concentrated stock solutions at -20 ºC.

Data analysis

Peak EPSC and IPSC amplitudes were measured using pClamp 8.2 software (Molecular Devices). Grouped data was plotted by expressing EPSC and IPSC amplitudes for each cell as a

percentage of the average amplitude of the baseline responses, plus or minus one standard error of the mean. The average amplitudes of EPSCs and IPSCs obtained during each set of recordings at 10 min intervals during the baseline, E2 application, and washout were compared using one-way repeated measures analysis of variance (ANOVAs) and planned comparison's using Holm's modified Bonferroni's *t*-test. The final wash recordings, during which estradiol was removed from the ACSF, were used to determine if any changes in synaptic currents induced by E2 were reversible. All data are reported as the mean plus or minus one standard error of the mean.

RESULTS

Recordings were obtained from a total of 29 neurons located in layers II and III of the lateral entorhinal cortex. The lateral entorhinal cortex contains fan cells that exhibit a hyperpolarization-induced sag (I_h) and fast afterhyperpolarization, pyramidal cells that lack I_h and minimal fast afterhyperpolarization, and multipolar cells that have intermediate properties (Tahvildari & Alonso, 2005). Neurons were characterized by monitoring membrane potential responses to positive and negative 500 ms current steps. The present study did not characterize the morphology of the recorded neurons, and the electrophysiological characteristics of lateral entorhinal cortex neurons are variable within neuron types. However, we tentatively classified 2 of 10 cells as pyramidal neurons in AMPA recordings, and 5 of 10 neurons as pyramidal neurons in GABA recordings. The use of Cs^{2+} and QX314 for recordings of NMDA receptor-mediated EPSCs prevented classification of those neurons. We found that putative pyramidal (Figure 3.1A) and fan (Figure 3.1B) neurons did not notably differ in the effects of E2 on EPSCs and IPSCs.

AMPA receptor-mediated EPSCs

The contribution of AMPA glutamate receptors to the facilitation of synaptic responses in lateral entorhinal cortex neurons was assessed in cells held at -70 mV in the presence of the NMDA receptor blocker APV (50 μM), the $GABA_A$ blocker picrotoxin (50 μM), and the $GABA_B$ blocker (1 μM) CGP. The group of 10 cells had a mean resting potential of neurons of -74.8 ± 1.3 mV, peak input resistance of 92.7 ± 18.4 MΩ, rectification ratio of 14.7 ± 4.9 %, and a holding current of -30.9 ± 33.6 pA at -85 mV. Baseline EPSC amplitude was 232.3 ± 44.3 pA in response to stimulation pulses of 138 ± 2 μA.

Application of 10 nM E2 for 20 min did not significantly affect the amplitude of AMPA-mediated EPSCs. Mean EPSC amplitude was 103.8 ± 3.5 % of baseline levels after 20 min of E2, and was 94.1 ± 3.3 % of baseline levels at the end of the washout period (Figure 3.2A_1). The change in EPSCs was not statistically significant (Figure 3.2A_2; n = 10, $F_{5,45} = 2.36$, p = 0.055, $\eta^2 = 0.208$). E2 has been shown to enhance AMPA-mediated responses in hippocampal neurons, but reliable increases have typically been observed in only about half of the recorded neurons (G. Z. Huang & Woolley, 2012; Smejkalova & Woolley, 2010; Wong & Moss, 1992). We found that only two entorhinal neurons, one putative pyramidal and one putative fan neuron, showed increases in EPSC amplitude more than 10% above baseline levels after 20 min of E2 application. Application of E2 therefore does not reliably increase AMPA receptor-mediated EPSCs in layer II/III entorhinal neurons.

NMDA receptor-mediated EPSCs

We tested here whether E2 can directly modulate pharmacologically isolated NMDA receptor-mediated EPSCs. Cells were recorded using a Cs^{2+}-based recording solution and held at +45.7 mV in the presence of the AMPA receptor blocker (50 μM) CNQX, the $GABA_A$ blocker picrotoxin (50 μM), and the $GABA_B$ blocker CGP (1 μM). The group of 9 cells had a mean resting potential of -48.3 ± 4.8 mV, peak input resistance of -111.1 ± 15.0 MΩ, rectification ratio of -8.9 ± 6.9, and a holding current of (516.11 ± 59.71 pA at 42.3 mV) 612.6 ± 119.1 pA at -79.3 mV. Baseline EPSC amplitude was 116.0 ± 34.6 pA, evoked by pulses of 158.4 ± 24.4 μA.

Electrically evoked EPSCs were significantly reduced after bath application of E2 for 20-min (n = 9, $F_{5,40} = 2.79$, $p < 0.05$, $\eta^2 = .197$). EPSC amplitudes decreased to 87.7 ± 4.8 % of baseline levels following E2 application, and 85.5 ± 4.5 % of baseline levels at the end of the washout period (Figure 3.2B_2). Planned comparisons of recordings taken at the end of each phase did not detect any significant differences between the end of baseline compared to E2 (p = 0.075) or washout (p = 0.26). Therefore, there was a significant reduction in EPSC amplitude during the course of the recordings, and the lowest mean amplitudes were observed during the washout period, and results do not definitively indicate if E2 results in a reduction in NMDA receptor-mediated currents.

$GABA_A$-mediated IPSCs

E2 has been shown to reduce $GABA_A$ receptor-mediated responses in the hippocampus (G. Z. Huang & Woolley, 2012; Tabatadze et al., 2015), and a reduction in $GABA_A$ inhibition could mediate the E2-induced facilitation of mixed excitatory responses we observed in the entorhinal cortex (Batallán Burrowes et al., 2021). We recorded pharmacologically isolated $GABA_A$-mediated responses by holding cells at -80 mV using a KCl-based intracellular recording solution. The group of 10 cells had a mean resting potential of neurons of -64.3 ± 1.0 mV, peak input resistance of 109.4 ± 6.8 MΩ, rectification ratio of 9.6 ± 2.3%. Cells were maintained at -83.2 mV with a holding current of -171.9 ± 25.1 pA. Baseline EPSC amplitude was 130.2 ± 21.4 pA using stimulation pulses of 355.5 ± 240.5 μA.

Application of E2 for 20 min resulted in a marked decrease in IPSC amplitude to during the drug period (Figure 3.3B), and there was a significant reduction in IPSC amplitudes to 68.3 ± 9.1 % of baseline levels after application of E2 for 20 min. The effect of E2 on IPSCs showed a partial reversal during the wash period, and amplitudes were 81.8 ± 6.6 % of baseline levels at the end of the washout period ($n = 10$, $F_{5,45} = 7.58$, $p < 0.001$, $\eta^2 = .457$). Planned comparisons showed that IPSC amplitudes were significantly reduced after 10- ($p = 0.007$) and 20-min application of E2 ($p < 0.001$), and during the first 10 min of the washout period ($p = 0.003$), but were not significantly different after 20 min of washout ($p = 0.027$). Therefore, application of E2 for 20 min results in a large, rapid, and reversible reduction in IPSC amplitude that could contribute to an enhancement of excitatory synaptic transmission in the entorhinal cortex.

DISCUSSION

Some of the cognitive effects of estrogens have been attributed to the modulation of excitatory synaptic transmission in the hippocampal formation, but changes in the entorhinal cortex could also contribute (Burwell, 2000; Evans, 2019; Finney, Shvetcov, et al., 2020). Previous findings from this lab have demonstrated that application of 17β-estradiol (E2) rapidly enhances evoked excitatory synaptic responses in layer II/III of the entorhinal cortex via activation of GPER1 receptors (Batallán Burrowes et al., 2021). The present study assessed the effect of E2 on pharmacologically isolated AMPA, NMDA and $GABA_A$ receptor-mediated postsynaptic currents. Results indicate that E2 did not have a significant effect on isolated AMPA receptor-mediated EPSCs, but the amplitudes of both NMDA receptor-mediated EPSCs and $GABA_A$-mediated IPSCs were markedly reduced. These results indicate that the facilitation

of excitatory synaptic strength in the entorhinal cortex observed previously, which reflects the combined effects of excitatory and inhibitory synaptic inputs onto entorhinal neurons, is likely caused by a reduction in synaptic inhibition onto principal neurons (Batallán Burrowes et al., 2021). The resulting facilitation of excitatory synaptic responses in the superficial layers of the entorhinal cortex may enhance neuronal processing within the entorhinal cortex and may also increase synaptic activation of the targets of entorhinal neurons in the hippocampal subfields (Kim et al., 2006). Changes in synaptic inhibition within the entorhinal cortex induced by estrogen may therefore contribute to changes in cognitive function that have previously been attributed to the actions of estrogen within the hippocampal formation.

AMPA and NMDA receptor-mediated EPSCs

The effects of estrogens on synaptic function in the hippocampus are varied and may be dependent on the subtype of ER that is activated. Extracellular electrophysiological recordings collected with multielectrode arrays show that E2 application can facilitate excitatory transmission in all hippocampal subfields, except for the CA2 region (Kim et al., 2006). Kumar et al. (2015)showed that application of E2 to hippocampal slices facilitates excitatory postsynaptic field potentials in the dentate gyrus. In their study, selective activation of each of the ER subtypes resulted in faciliatory effects, however, activation of GPER1 receptors resulted in a greater facilitation than either ERα or ERβ alone (Kumar et al., 2015). Whole-cell recordings from CA1 neurons has also shown significant facilitation of excitatory transmission following application of E2 or selective activation of GPER1 and ERβ in females (Oberlander & Woolley, 2016).

However, facilitatory effects are not expressed in all neurons, and application of E2 has been found to rapidly increase EPSPs or EPSCs in approximately half of the recorded neurons. For example, Wong and Moss (1992) found that 35 of 64 neurons showed increases in AMPA-mediated responses. Similarly, Smejkalova and Woolley (2010) found that AMPA receptor-mediated EPSCs showed a 20% or more facilitation in only 40% of cells (See also Foy et al., 1999). In the present study, E2 may have caused increased AMPA mediated responses in some neurons, as 3 of 10 cells showed increases in AMPA receptor-mediated EPSCs greater than 20% after 10 or 20 min of E2 application. However, the 3 neurons included both putatively identified pyramidal and fan cells. It is also unclear if these changes were spontaneous or induced by

application of E2. Therefore, it is possible that E2 may induce increases in isolated AMPA receptor-mediated EPSCs in the entorhinal cortex in some neuron types, but the present evidence does not provide strong support for this idea.

The amplitude of isolated NMDA receptor-mediated EPSCs was reduced in the present study following application of E2 for 20 min, and the responses remained reduced throughout the duration of the wash period. A similar change in NMDA receptor-mediated synaptic currents can therefore not explain the reversible *increase* in field EPSPs induced by E2 in the entorhinal cortex previously reported (Batallán Burrowes et al., 2021). Control recordings to monitor possible changes in NMDA responses without application of E2 were not conducted in the present study; therefore, it is possible that the reduction in amplitude of NMDA responses was not due to E2, but rather due to recording conditions and the passage of time. A Cs^{2+}-based intracellular recording solution was used to record NMDAR-mediated responses from neurons and the mean input resistance gradually increased during the recording period. The increase in input resistance may be due to a slow increase in the effectiveness of Cs^{2+} in blocking potassium conductances as the Cs^{2+} diffused from the soma region to more distal dendrites. The increase in input resistance suggests that the reduction in NMDA-mediated currents was not due to a reduction in input resistance or health of the recorded neurons.

The reduction in NMDA receptor-mediated EPSCs observed here could be associated with an ERβ-mediated reduction in NMDA receptor activation. Hippocampal application of E2 at concentrations higher than 7 nM have been found to rapidly suppress NMDA-mediated EPSCs for prolonged periods, and application of the ERβ agonist DPN (5 nM) results in a similar suppression of NMDA-mediated EPSCs (Tanaka & Sokabe, 2013). This could be tested in the entorhinal cortex by repeating recordings to determine if the suppression of NMDA responses induced by E2 is blocked during concurrent application of the ERβ antagonist ICI 182,780. Further, in contrast to the reduction in NMDA-mediated EPSCs observed here during application of 10 nM E2, it is possible that NMDA responses in the entorhinal cortex might be *enhanced* if we applied a lower concentration of E2. The activation of ERα by 1 nM estradiol benzoate has been shown to result in a reversible facilitation of pharmacologically isolated NMDA EPSPs in hippocampal CA1 neurons (Tanaka & Sokabe, 2013). However, activation of ERα is unlikely to induce large effects because ERα expression is relatively sparse in the entorhinal cortex and is localized to the deep layers V and VI (Kritzer, 2002). This could be investigated by repeating

experiments with 1 nM E2, or with the ERα agonist PPT, which might result in a potentiation of NMDA-mediated EPSCs.

Modulation of $GABA_A$-mediated IPSCs

Application of E2 resulted in a strong and reversible decrease in the amplitude of pharmacologically isolated $GABA_A$-mediated IPSCs. The reduction of inhibitory synaptic transmission induced by E2 observed here likely mediates the reversible facilitation of mixed excitatory field EPSPs that we observed in response to the same concentration of E2 in layer II/III of the entorhinal cortex (Batallán Burrowes et al., 2021). The amplitude of field EPSPs is determined by the summation of simultaneous excitatory and inhibitory synaptic inputs, and a reduction in the strength of inhibitory synaptic input can increase the amplitude of evoked excitatory synaptic responses (Hamam et al., 2007). Other studies have also shown that E2 can enhance synaptic excitability by suppressing inhibitory synaptic transmission. In cultured hippocampal neurons, estrogen reduces GABAergic transmission and this depolarizes neurons which results in an increase in dendritic spine density due to enhanced voltage-dependent activation (Murphy et al., 1998).

Estrogens can affect synaptic inhibition through multiple mechanisms. E2 has been found to reduce the time that GABA receptors spend at inhibitory synapses in cultured cortical neurons (Mukherjee et al., 2017). Application of E2 in the CA1 region has also been observed to reduce the amplitude of synaptically evoked IPSCs, miniature IPSCs, and spontaneous IPSCs, and a reduction in the frequency of miniature IPSCs suggests that these effects were due to a reduction in the probability of presynaptic GABA release (Rudick et al., 2003). Our previous findings that the E2-induced facilitation of fEPSPs in the entorhinal cortex is dependent on activation of GPER1 receptors suggests that the inhibition of IPSCs observed here is also due to activation of GPER1 receptors (Batallán Burrowes et al., 2021). GPER1 has been found to increases synaptic inhibition in the basolateral amygdala (Tian et al., 2013), and a reduction of miniature IPSC frequency induced by E2 in the prefrontal cortex is blocked by ERβ and GPER1 antagonists, but not by an ERα antagonist (Doncheck et al., 2021). In the CA1 region, application of E2 can rapidly supress inhibitory transmission via activation of ERα, which stimulates postsynaptic $mGluR_1$-dependent mobilization and release of anandamide. Anandamide in turn binds to presynaptic cannabinoid receptors of inhibitory synapses and inhibits the release of GABA (G.

Z. Huang & Woolley, 2012; Tabatadze et al., 2015). However, the entorhinal cortex shows minimal expression of ERα (Kritzer, 2002), thus the reduction of IPSCs in the entorhinal cortex does not likely depend on ERα activation.

Further experiments could investigate if GPER1 receptors mediate the reduction in IPSCs by determining if the GPER1 antagonist G15 blocks the effects of E2 on IPSCs. If GPER1 receptors are determined to mediate the suppression of $GABA_A$-mediated IPSCs, then additional experiments could determine the intracellular signalling pathway through which GPER1 receptors modulate inhibitory synapses. GPER1 receptors have been linked to multiple intracellular signalling pathways in different brain areas. Activation of GPER1 receptors can modulate adenylyl cyclase and cAMP signaling, can increase intracellular calcium-signaling via production of IP_3, and can also affect AKT and MAPK signalling (Alexander et al., 2017; Finney, Shvetcov, et al., 2020; Lu & Herndon, 2017). The reduction of inhibitory synaptic responses induced by E2 in the hippocampus results from an IP_3 and calcium-dependent activation of anandamide which suppress GABA release from inhibitory terminals (G. Z. Huang & Woolley, 2012; Tabatadze et al., 2015). It is possible that GPER1 receptor-mediated changes to calcium-signaling could activate similar mechanisms in entorhinal cortex neurons (Alexander et al., 2017; Brailoiu et al., 2007). Experiments that use of blockers of second messenger pathways in the intracellular recording solution could determine which pathways link GPER1 receptors to postsynaptic alterations in GABA receptors.

Functional implications

Estrogens are believed to affect hippocampal function through both genomic and rapid non-genomic effects (Taxier et al., 2020) and E2 is also known to rapidly facilitate synaptic transmission in the entorhinal cortex (Batallán Burrowes et al., 2021). The present results indicate that the rapid facilitation of excitatory synaptic transmission in the entorhinal cortex is likely due to a reduction in the strength of inhibitory synaptic transmission. Understanding of the effects of estrogens on synaptic transmission and intracellular pathways are important to understanding the underlying synaptic mechanisms in the entorhinal cortex that may contribute to the enhancing effects of estrogen on cognition (Van Cauter et al., 2013; Deshmukh & Knierim, 2011; Knierim et al., 2013). The findings of this study could also help clarify the neurobiological mechanisms that underlie shifts in cognitive strategies that occur as a function of

changes in estrogen levels during the menstrual cycle and in the transition to menopause (Engler-Chiurazzi et al., 2017). GPER1 receptors are believed to be linked with the neuroprotective effects of estrogens (Alexander et al., 2017), and an understanding of the effects of estrogen on synaptic transmission could be applied to other fields of research including dementia-related cognitive decline as seen in Alzheimer's disease which has been linked to reductions in estrogen when controlling for age (Frick & Kim, 2018).

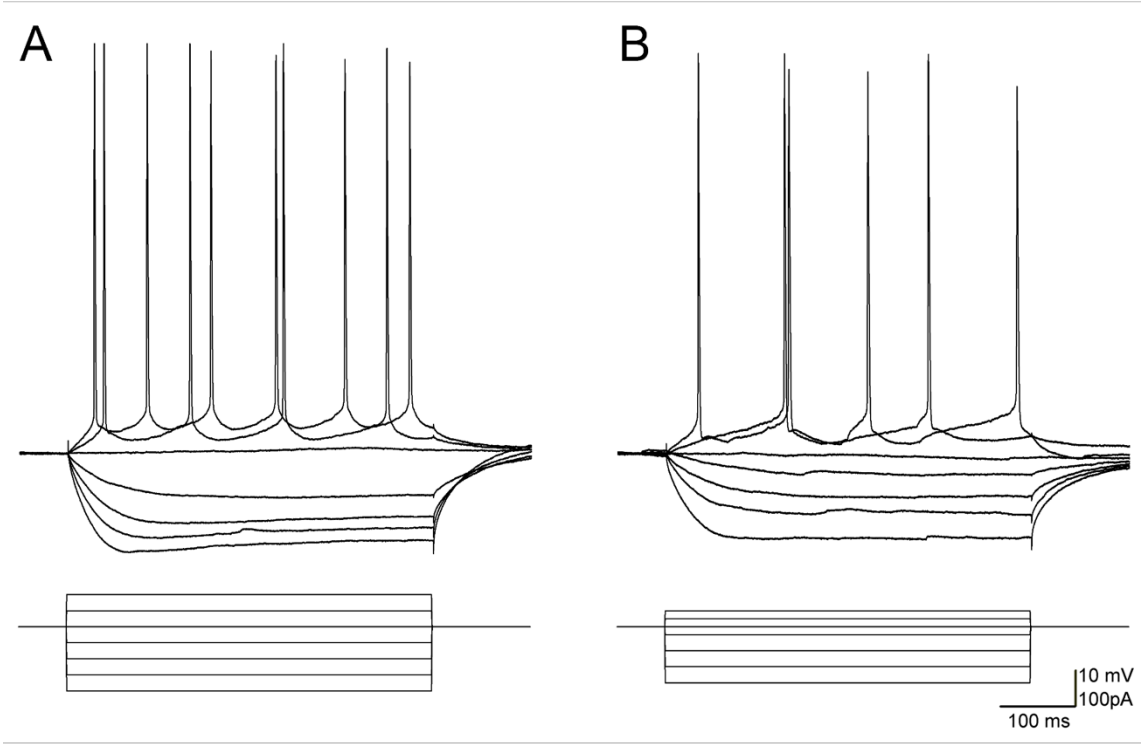
A
B
10 mV
100pA
100 ms

Figure 3.1. Representative traces of membrane potential responses to current-step injections in a putative pyramidal cell using K-gluconate-based patch solution (A) and a fan cell using KCl-based patch solution (B). Current steps 500 ms in duration were injected at levels ranging from -200 pA to 150 pA (see lower traces) in layer II/III lateral entorhinal neurons in normal ACSF using intracellular recording solutions used in experiments to measure either AMPAR-mediated EPSCs or $GABA_AR$-mediated IPSCs. Membrane potential responses were used to characterize electrophysiological profiles of neurons and to tentatively classify them as pyramidal cells or fan cells. Note the presence of a sag in the voltage response to strong negative current steps in the fan cell (A) regular firing in the pyramidal cell (B).

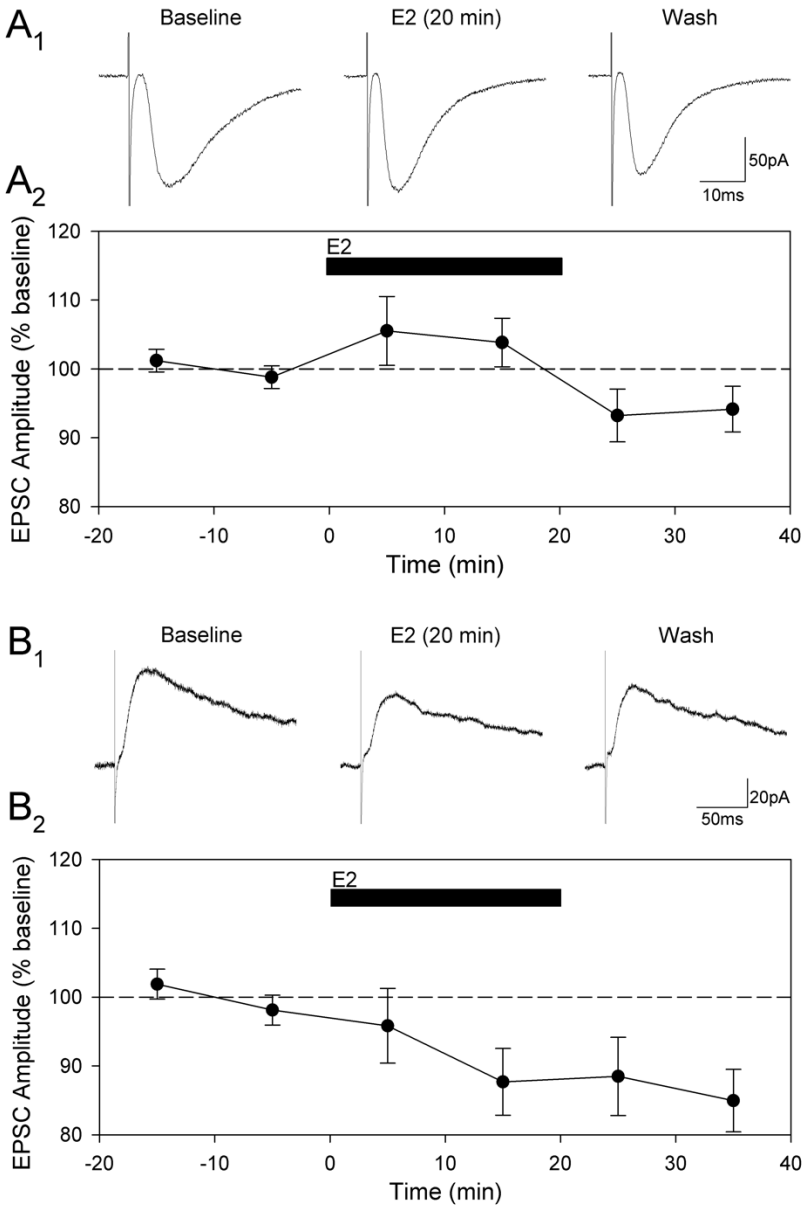
A1
Baseline
E2 (20 min)
Wash
50pA
10ms
A2
E2
EPSC Amplitude (% baseline)
120
110
100
90
80
-20
-10
0
10
20
30
40
Time (min)
B1
Baseline
E2 (20 min)
Wash
20pA
50ms
B2
E2
EPSC Amplitude (% baseline)
120
110
100
90
80
-20
-10
0
10
20
30
40
Time (min)

Figure 3.2. Bath-application of 10 nM 17β-estradiol (E2) for 20 min (black bar) had no significant effect on pharmacologically isolated AMPA-mediated EPSCs in layer II/III lateral entorhinal cortex neurons evoked by layer I stimulation. 17β-estradiol application was associated with a reduction in the amplitude of pharmacologically isolated NMDA-mediated EPSCs. **A.** Traces show pharmacologically isolated AMPAR-mediated EPSCs recorded from a representative cell. Traces are averages of five consecutive responses obtained at the end of each experimental period (A_1). Group averages of EPSC amplitudes (± 1 SEM) were obtained at 10-min intervals during baseline, application of 17β-estradiol (black bar), and the washout period (n = 10; A_2). Averages are represented as percent changes from baseline values. **B.** Representative averaged traces of pharmacologically isolated NMDAR-mediated EPSCs in a representative neuron (B_1). Mean EPSC amplitudes in the group of neurons tested (n=9; B_2) are represented as percent changes from baseline values.

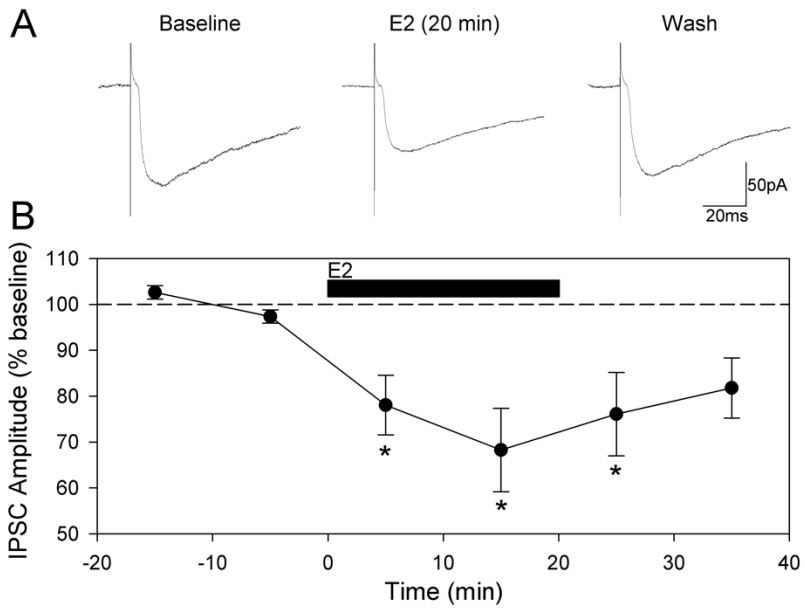
A
Baseline
E2 (20 min)
Wash
50pA
20ms
B
IPSC Amplitude (% baseline)
110
100
90
80
70
60
50
E2
*
*
*
-20
-10
0
10
20
30
40
Time (min)

Figure 3.3. Bath application of 10 nM 17β-estradiol for 20 min significantly, and reversibly, reduced pharmacologically isolated $GABA_AR$-mediated IPSCs. Representative averaged pharmacologically isolated $GABA_AR$-mediated IPSCs recorded from a representative cell are based on averages of five consecutive responses obtained at the end of each experimental period (A). Group averages of IPSC amplitudes (± 1 SEM) are shown at 10-min intervals during the baseline period, application of 17β-estradiol (black bar), and the washout period (n = 10; B). Averages are represented as percent changes from baseline values and significant changes are indicated by an asterisk ($p < .01$).

CHAPTER 4

OVARIECTOMY REDUCES CHOLINERGIC MODULATION OF EXCITATORY SYNAPTIC TRANSMISSION IN THE RAT ENTORHINAL CORTEX.

ABSTRACT

Estrogens are thought to contribute to cognitive function in part by promoting the function of basal forebrain cholinergic neurons that project to the hippocampus and cortical regions including the entorhinal cortex. Reductions in estrogens may alter cognition by reducing the function of cholinergic inputs to both the hippocampus and entorhinal cortex. In the present study, we assessed the effects of ovariectomy on proteins associated with cholinergic synapses in the entorhinal cortex. Ovariectomy was conducted at PD63, and tissue was obtained on PD84 to 89 to quantify changes in the degradative enzyme acetylcholinesterase, the vesicular acetylcholine transporter, and muscarinic M_1 receptor protein. Although the vesicular acetylcholine transporter was unaffected, ovariectomy reduced both acetylcholinesterase and M_1 receptor protein, and these reductions were prevented by chronic replacement of 17β-estradiol following ovariectomy. We also assessed the effects of ovariectomy on the cholinergic modulation of excitatory transmission, by comparing the effects of the acetylcholinesterase inhibitor eserine on evoked excitatory synaptic field potentials in brain slices obtained from intact rats, and from ovariectomized rats with or without 17β-estradiol replacement. Eserine is known to prolong the effects of endogenously released acetylcholine, resulting in an M_1-like mediated reduction of glutamate release at excitatory synapses. The reduction in excitatory synaptic potentials in layer II of the entorhinal cortex induced by 15-min application of 10 μM eserine was greatly reduced in slices from ovariectomized rats as compared to intact rats and ovariectomized rats with replacement of 17β-estradiol. The reduced modulatory effect of eserine is consistent with the observed changes in cholinergic proteins, and suggests that reductions in 17β-estradiol following ovariectomy lead to impaired cholinergic function within the entorhinal cortex.

Estrogens are produced by the ovaries and synthesized in the brain, and are known to modulate cognitive functions in both humans and animals (Finney, Proschogo, et al., 2020; Hussain et al., 2014; Taxier et al., 2020). Cyclic increases of estrogens are associated with improved attention and recollection of verbal, emotional and spatial memory in humans (Hussain et al., 2016; Newhouse & Dumas, 2015; Nielsen et al., 2013; Zoladz et al., 2015) and rats show improved object recognition and working memory during proestrus when estrogens are high (Van Goethem et al., 2012; Pompili et al., 2010). Administration of estrogens also enhance working memory and hippocampal-dependent tasks including spatial navigation, novel object discrimination, and social learning (Daniel et al., 1997; Jacome et al., 2010), and can promote the use of spatial cognitive strategies associated with the hippocampus (Almey et al., 2014; Korol et al., 2004; Quinlan et al., 2008).

Estrogens are thought to promote cognitive function by enhancing excitatory synaptic transmission though multiple mechanisms in the hippocampus and other cortical regions including the entorhinal cortex (Arevalo et al., 2015; Batallán Burrowes et al., 2021; Spencer et al., 2008; Wong & Moss, 1992). In the hippocampal CA1 region, 17β-estradiol (E2) reduces inhibitory synaptic transmission (Tabatadze et al., 2015), facilitates excitatory transmission (Foy et al., 1999; Kumar et al., 2015; Oberlander & Woolley, 2016), and increases dendritic spine density (Woolley & McEwen, 1993). We have also found that activation of G-protein estrogen receptor-1 (GPER1) receptors by E2 induces a rapid and reversible potentiation of excitatory synaptic responses in the entorhinal cortex (Batallán Burrowes et al., 2021), indicating that E2 can facilitate excitatory synaptic inputs to the entorhinal cortex. Estrogens are also thought to enhance cognition by promoting the function of basal forebrain cholinergic neurons. Basal forebrain cholinergic neurons contain estrogen receptor α (Hammond et al., 2011; Shughrue &

Merchenthaler, 2001), and the transcription of choline acetyltransferase is modulated during the estrous cycle, and enhanced by E2 following ovariectomy (Gibbs, 1996; McMillan et al., 1996). Cholinergic inputs to the hippocampus and entorhinal cortex promote neuronal synchronization and reduce excitatory transmission (Barrett & Chapman, 2013; Heys et al., 2012; Nuñez & Buño, 2021), and play central roles in attention, sensory processing, learning and memory, and spatial navigation (Gibbs, 2010; Newhouse & Dumas, 2015; Ping et al., 2008; Solari & Hangya, 2018; Spencer et al., 2008).

Reductions in cholinergic function in the hippocampal region associated with a decline in circulating estrogens are thought to contribute to cognitive changes during the perimenopause transition (Newhouse & Dumas, 2015). Surgical removal of ovaries is associated with cognitive decline in women that is prevented by hormone replacement therapies containing estrogens (Hara et al., 2015; Phillips & Sherwin, 1992). Ovariectomy in rats results in estrogen-dependent changes in cognitive function that are due in part to disruptions in cholinergic transmission (Gibbs, 2010; Singh et al., 1994). Ovariectomy results in estrogen-dependent reductions in the density of basal forebrain cholinergic neurons and reduces cholinergic terminals and acetylcholine release in the hippocampus (Gibbs, 1997; Hammond et al., 2011). This can result in reduced activation of NMDA glutamate receptors and the impairment of hippocampal function (Daniel & Dohanich, 2001; Hammond & Gibbs, 2011).

A reduction in cholinergic function following ovariectomy in the entorhinal cortex could have substantial effects on cognition by affecting both excitatory synaptic transmission and neuronal excitability (Heys et al., 2012). Reduced cholinergic function could increase basal excitatory transmission by reducing the inhibition of glutamate release caused by acetylcholine (Barrett & Chapman, 2013), and could also reduce neuronal excitability because acetylcholine

normally depolarizes the membrane potential of entorhinal neurons (Klink & Alonso, 1997; Shalinsky et al., 2002). In addition, the expression of theta- and gamma-frequency rhythmic population activities that contribute to mnemonic processing and spatial navigation are likely to be reduced because these rhythms are dependent on muscarinic depolarization of principal neurons and activation of GABA interneurons that synchronize population rhythms (Dannenberg et al., 2016; Heys et al., 2012).

To assess the effects of ovariectomy on cholinergic function in the entorhinal cortex, we measured the expression of proteins associated with cholinergic transmission three weeks following either a sham surgery, ovariectomy, or ovariectomy with chronic replacement of E2 (Almey et al., 2013; Mannino et al., 2005). Entorhinal tissue samples were quantified for expression of the degradative enzyme acetylcholinesterase, the vesicular acetylcholine transporter (VAChT), and M_1 muscarinic receptor protein. The effects of ovariectomy on the cholinergic modulation of excitatory synaptic transmission were also assessed by quantifying the effect of the acetylcholinesterase inhibitor eserine on excitatory postsynaptic field potentials in vitro. Evoked excitatory synaptic responses are reduced by activation of M_1-like muscarinic receptors in the entorhinal cortex (Barrett & Chapman, 2013; Hamam et al., 2007), and a smaller eserine-induced reduction in synaptic responses could occur if cholinergic function is compromised by ovariectomy.

METHODS

Subjects and surgery

Experiments were conducted according to the guidelines of the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee (Permit Number: 30000253). Female Long-Evans rats (Charles River) were pair-

housed under a 12-hour light-dark light cycle. Animals that received ovariectomies underwent surgery on PD63. Rats were anaesthetized with 3 % isoflurane in O_2 and ovaries were removed via a single 2 cm lumbar skin incision and bilateral tears in the abdominal musculature. Groups of animals received either a sham surgery in which only the skin incision was made, ovariectomy, or ovariectomy and immediate implantation of a subcutaneous capsule in the nape of the neck to maintain low circulating levels of 17β-estradiol (E2; Mannino et al., 2005). Capsules were made using the materials and methods of Almey et al. (2013) which have resulted in serum E2 of 38 pg/ml 7 to 14 days following ovariectomy, and 29 pg/ml 21 days following ovariectomy (Almey et al., 2013). Silastic tubing (1 cm-long; Dow Corning, I.D. 1.47 mm, O.D. 1.96 mm) sealed with silicone contained 8 mg of 5 % cyclodextrin-encapsulated 17β-estradiol (Sigma Aldrich) in cholesterol (Bioshop Canada). Animals received postsurgical injections of buprenorphine (0.05 mg/kg, s.c.) every 12 hours for 72 hours.

Protein quantification

Tissue preparation

Tissue was prepared for protein quantification 21 to 26 days following surgery. Subjects were anaesthetized with isoflurane and brains were rapidly removed and cooled (4°C) in oxygenated (95% O_2 and 5% CO_2) high sucrose ASCF solution containing, in mM, 2 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 250 sucrose, 10 dextrose and 0.5 $CaCl_2$. Horizontal slices (400 μm) were obtained using a vibratome (Leica, VT1200), and were then kept at 32 °C for 30 min in normal ACSF containing 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose. The medial and lateral entorhinal cortex were isolated through the dorso-ventral extent of the brain (Olajide & Chapman, 2021; Paxinos & Watson, 1998). Tissue was placed in normal ACSF consisting (in mM) of 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26

$NaHCO_3$, and 10 dextrose saturated with 95% O_2 and 5% CO_2 at 32 °C for 30 min. Tissue was then kept in normal ACSF at 22 - 24 °C for 30 min. Massachusetts

Protein extraction and western blotting

Tissue was collected into microfuge tubes and snap-frozen in liquid nitrogen. Tissues were then disrupted in radioimmunoprecipitation assay homogenization buffer (50 mM Tris, pH 7.4, 0.1% SDS, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM PMSF) using a tissue sonicator (QSonica, Q55). The quantity of protein in each sample was determined using BCA Protein Assay (Thermo Fisher, 23227) and an ELISA Fluorostar Analysis System plate reader. Bovine serum albumin (BSA) was used as the standard for protein quantification. Protein samples (20 to 40 µg) were resolved on Tris-glycine 8 - 10 % SDS-PAGE gels. The resolved proteins were transferred from gels to nitrocellulose membrane (Bio-Rad, 1620112) and blocked for 1 hour in either 5% milk or 5% BSA, depending on the specific antibody, in Tris-buffered saline (TBS) containing 0.2% Tween-20 (TBST).

Primary antibodies including anti-choline acetylcholinesterase (1:1000; Abcam, AB183591), anti-vesicular acetylcholine transporter (1:2000; Abcam, AB235201), anti-cholinergic muscarinic receptor 1 (1:2000; Alomone labs, AMR-010), anti-vinculin (1:4000; Abcam, AB130007), anti-β-Actin (1:5000; Abcam, AB8226), were diluted in TBST containing either of 5% milk or 5% BSA and incubated overnight at 4 °C. Membranes were then washed 3 times for 5 minutes each in TBST and incubated at room temperature with either peroxidase-conjugated goat anti-mouse secondary antibody (used at 1: 3000 for anti-vinculin, and 1: 5000 for anti-β-Actin; Millipore Sigma, AP124P) or peroxidase-conjugated goat anti-rabbit secondary antibody (used at 1: 5000 for anti-acetylcholinesterase, 1: 6000 for anti-vesicular acetylcholine transporter, and 1:4000 for anti-cholinergic muscarinic receptor 1; Millipore Sigma, AP132P) for 1- 2 hours. Immunoreactivity was detected using ECL Western blotting substrate

(Thermoscientific, 32106) and visualized using a CDP-STAR chemiluminescence system (Amersham hyperfilm ECL). Western blot data were compiled from at least six animals, and bands were quantified by densitometric analysis using Image-J software (version 1.41).

Results were analysed using GraphPad Prism software version 8.0.1 and analyzed with a mixed design Group (Intact, OVX, OVX+E) by Site (MEC vs LEC) analyses of variance (ANOVA) and Tukey comparisons. Antibody signals were normalized against the largest loading control immunoreactivity recorded in the medial or lateral entorhinal cortex of rats that received sham surgery. Bar graphs indicate the mean and standard error of the mean, normalized to the largest control value in percentage, and include values obtained from individual animals.

Electrophysiological recordings

Recordings were obtained 7 to 17 days after ovariectomy on PD70 to PD80, and conducted during the dark phase of the light-dark cycle. Horizontal brain slices (400 µm-thick) containing the hippocampal and entorhinal regions were obtained and maintained for at least 30 min at 22-24 ºC prior to recordings. Slices were placed on a nylon net in a gas-fluid interface recording chamber (Fine Science Tools) and perfused at 2.0 ml/min with the upper surfaces exposed to humidified 95% O_2, 5% CO_2 atmosphere. Field excitatory postsynaptic potentials (fEPSPs) were recorded using borosilicate glass pipettes (1.0 mm OD; Sutter Model P97) filled with ACSF (4 to 6 MΩ). Electrodes were positioned 75 to 200 µm below the surface of the slice in layer I of the lateral entorhinal cortex, close to the layer II border, using a dissecting microscope (Leica, MS5). Bipolar stimulating electrodes made from tungsten electrodes (0.8 - 1 MΩ, FHC Inc.) were placed in the middle of layer I, 0.3 to 0.4 mm rostral to the recording electrode.

Changes in fEPSPs induced by the acetylcholinesterase inhibitor eserine hemisulfate (Sigma) were compared in slices obtained from intact rats and ovariectomized rats with and without E2 capsules. Constant current pulses (0.1 ms duration) were delivered every 30 seconds with a stimulus generator and stimulus isolation unit (WPI, A300 and A365), using a current that elicited fEPSPs approximately 65% of the maximal response (50 -135 μA). Field EPSPs were amplified (DC-3 kHz; Axoclamp 2B, Molecular Devices) and digitized (20 kHz; Digidata 1322A) using Clampex 8.2 software (Molecular Devices). In each slice, after a baseline period of at least 10 min in which fEPSP amplitude varied less than 10%, 10 μM eserine was added to the ACSF for a period of 15 minutes. There was then a 40 min follow-up period in normal ACSF. Peak amplitudes of fEPSPs were measured using pClamp 8.2 software (Molecular Devices), and data for each slice were expressed as a percent of the average baseline fEPSP amplitude. The average fEPSP amplitude obtained during the baseline period, and 25 - 30 and 50 - 55 min after the onset of eserine, were assessed using a mixed design ANOVA with Group (Intact, OVX, OVX+E) and Time (baseline, 25-30 min, 50-55 min) as factors. Significant effects were investigated with post-hoc Tukey's comparisons.

RESULTS

Protein quantification

Western blot quantification was used to measure proteins related to cholinergic function, and to determine if changes in protein expression induced by ovariectomy could be blocked by chronic E2 administration (n=6 per group). In comparison to tissue from animals that received sham surgery, ovariectomy resulted in marked reductions in AChE protein immunoexpression ($F_{2, 20}$= 16.54, $p<0.0001$) in both the medial ($p=0.003$) and lateral entorhinal cortex ($p=0.002$) (Fig 4.1A). Further, the replacement of E2 following ovariectomy prevented significant

reductions in AChE in both the medial (p=0.208) and lateral entorhinal cortex (p=0.821). Differences in AChE in ovariectomized rats versus ovariectomized rats with E2 replacement were significant for the lateral (p=0.007), but not medial entorhinal cortex (p=0.113). Ovariectomy therefore causes a reduction in AChE protein in the entorhinal cortex that is prevented by chronic administration of E2. Although the vesicular acetylcholine transporter (VAChT) is expressed broadly and serves as a marker for cholinergic axon terminals (Ichikawa et al., 1997), we found that ovariectomy did not significantly affect expression of VAChT. Results showed no significant main effect of group ($F_{1, 30} = 0.15$, p=0.706) on VAChT expression (Fig 4.1B).

Ovariectomy resulted in a strong reduction in M_1 muscarinic receptor protein expression in both the medial and lateral entorhinal cortex in comparison to the sham control group ($F_{2,20} = 27.22$, p<0.0001; medial, p=0.001; lateral, p<0.0001). Replacement of E2 prevented the reduction in M_1 receptor protein. There was no significant difference in M_1 receptor expression between control animals and ovariectomized animals that received E2 replacement (medial, p=0.660; lateral, p=0.946). Muscarinic M_1 expression was also significantly reduced in ovariectomized animals in comparison with ovariectomized animals that received E2 replacement (medial, p=0.009; lateral, p<0.001) (Fig 4.1C). Ovariectomy therefore causes reductions in M_1 protein in both the medial and lateral entorhinal cortex that are prevented by chronic administration of E2.

Effects of eserine on field EPSPs

Application of eserine resulted in reductions in the amplitude of field excitatory postsynaptic potentials (fEPSPs) that began about 10 min after the onset of eserine application, and were maximal after approximately 25 to 30 min during the wash period. In intact rats, EPSP

amplitude was reduced to 79.1 ± 2.2 % of baseline levels 25-30 min after the onset of the drug, and to 83.0 ± 3.2 % at the end of the recording period (n=13 slices from 7 rats; Fig 4.2A). The size of the reduction was smaller in ovariectomized animals (11.5 ± 2.9 % versus 20.9 ± 2.2 % in intact rats), and fEPSP amplitudes in slices from ovariectomized animals were at 88.1 ± 3.2 % of baseline 25-30 min after onset of eserine, and 91.7 ± 3.5 % of baseline at the end of the recording period (n = 10 slices from 6 rats; Fig 4.2B). Ovariectomized rats that received E2 replacement showed declines in fEPSP amplitude similar to intact rats, and fEPSP amplitudes were 78.9 ± 2.5 % of baseline 25-30 min after onset of eserine, and 77.8 ± 3.1 % of baseline at the end of the recording period (n = 10 slices from 5 rats; Fig 4.2C). An ANOVA showed a significant group by time interaction ($F_{4,60}$=3.51, p=0.023). Tukey's comparisons indicated that, although reductions in fEPSPs did not differ between intact rats and ovariectomized rats that received E2 replacement (25-30 min, p = 0.997; 50-55 min, p = 0.296), slices from ovariectomized rats showed significantly smaller fEPSPs as compared to intact rats (25-30 min, p = 0.028; 50-55 min, p = 0.034), and as compared to ovariectomized rats with E2 replacement (25-30 min, p = 0.034; 50-55 min, p < 0.001). Therefore, the reduction in fEPSP amplitude induced by eserine was significantly smaller in slices from ovariectomized animals versus intact rats, and this effect was prevented by replacement of E2 following ovariectomy.

DISCUSSION

Basal forebrain cholinergic neurons impact cognitive processing by modulating neuronal excitability and synaptic transmission in both the hippocampus and entorhinal cortex (Barrett & Chapman, 2013; Hamam et al., 2007; Klink & Alonso, 1997). Reductions in estrogens following ovariectomy are thought to impair cognitive processes in part by reducing the function of cholinergic afferents to the hippocampus (Hammond & Gibbs, 2011; Spencer et al., 2008), but

reduced cholinergic input to the entorhinal cortex is also likely to impact cognitive function. We have found that ovariectomy results in reductions in both acetylcholinesterase (AChE) and M_1 receptor protein in the medial and lateral entorhinal cortex, and that these reductions are prevented by chronic replacement of 17β-estradiol (E2). Ovariectomy also reduced the modulatory effects of the acetylcholinesterase inhibitor eserine on excitatory synaptic responses in the entorhinal cortex in vitro. Eserine induced a smaller reduction in the amplitude of field EPSPs in slices from ovariectomized vs intact rats, indicating a reduced cholinergic suppression of excitatory synaptic transmission (Barrett & Chapman, 2013), and this effect was prevented by chronic replacement of E2 following ovariectomy. This suggests that reductions in E2 following ovariectomy result in a functional impairment of cholinergic transmission in the entorhinal cortex.

The cognitive changes following ovariectomy that have been attributed to reduced basal forebrain cholinergic input to the hippocampus may be due in part to reduced cholinergic function in the medial and lateral entorhinal cortex (Hammond & Gibbs, 2011; Pala et al., 2019). The medial entorhinal cortex contributes to navigation and spatial processing and memory (Hafting et al., 2005) and the lateral entorhinal cortex is involved in olfaction, object recognition, and memory for object location (Knierim et al., 2013; Nilssen et al., 2019). We have shown that ovariectomy reduced AChE and M_1 receptor protein in both regions, and this may affect both local processing and the activity of entorhinal projections to the hippocampus (Burwell, 2000).

Reductions in cholinergic synaptic proteins

The finding that ovariectomy reduced AChE expression in entorhinal tissue is consistent with studies that have found reductions in ChAT mRNA in basal forebrain cholinergic nuclei (Gibbs, 1998; Gibbs et al., 1994; Luine, 1985). Ovariectomy also results in widespread

reductions in cholinergic projections to the hippocampus, prefrontal cortex, and olfactory bulbs as reflected by reductions in ChAT and high affinity choline uptake (Gibbs, 2000; Singh et al., 1994; Tinkler et al., 2004). We found that the reduction in AChE in the entorhinal cortex of ovariectomized animals was prevented by maintaining circulating E2 using a subcutaneous implant (Almey et al., 2013). Similarly, E2 replacement prevents reductions in cholinergic staining in hippocampus (Gibbs, 2000; Singh et al., 1994) and prefrontal cortex (Tinkler et al., 2004). It is not clear why levels of the vesicular acetylcholine transporter (VAChT) remained stable (Uzum et al., 2016) while levels of AChE were reduced by ovariectomy, but this suggests that ovariectomy resulted in a reduction in the function of cholinergic terminals without substantial loss of synaptic terminals or vesicular machinery.

Muscarinic M_1 receptor protein was reduced in the entorhinal cortex by ovariectomy, and this reduction was prevented by replacement of E2. These findings are consistent with the concurrent decrease in AChE protein, and with studies demonstrating reduced cholinergic function following ovariectomy (Gibbs, 2000; Singh et al., 1994; Tinkler et al., 2004). Ovariectomy decreases M_1 receptor mRNA in the hippocampus (Pala et al., 2019) and has been found to reduce M_1 and M_2 receptor binding in basal forebrain and cortical projection areas including the entorhinal cortex, although this effect was not prevented by E2 supplementation (Vaucher et al., 2002). In contrast, others have found that M_1 to M_5 receptor protein is increased 15 days following ovariectomy in the hippocampus and that these increases are prevented by E2 supplementation (Cardoso et al., 2010; El-Bakri et al., 2002). An increase in muscarinic M_4 receptors on glutamatergic terminals may also underlie reduced glutamate release in the hippocampus following ovariectomy (Stelly et al., 2012). Increases in muscarinic receptors following estrogen supplementation are also thought to contribute importantly to the cognitive

benefits of estrogen supplementation (Gibbs, 1999). Compared to their non-treated counterparts, postmenopausal women that receive hormone replacement therapy have increased plasma estradiol and higher muscarinic receptor densities in the striatum, hippocampus and frontal cortex (Norbury et al., 2007). Estrogen replacement following ovariectomy also results in an enhancement of hippocampal long-term synaptic potentiation that is dependent on muscarinic receptors (Stelly et al., 2012). Differences in the effects of ovariectomy on muscarinic receptors may be related to experimental variables including duration of ovariectomy and brain region examined.

Reduced effect of eserine on EPSPs

Reductions in fEPSP amplitudes began approximately 10 minutes after the onset of eserine application and persisted for the duration of recordings. The delayed onset of the effect is likely due to time needed for drug concentration to rise in our high-volume interface recording chamber, and for eserine to increase acetylcholine availability by blocking degradation of endogenously released acetylcholine. The reduction in fEPSPs did not reverse entirely; this may be due to the high binding affinity of eserine (Triggle et al., 1998), but eserine can also induce a long-term depression of synaptic responses in the CA1 region via a lasting reduction in glutamate release (Mans et al., 2014), and this may have contributed to the duration of the effect.

Electrophysiological results obtained here are consistent with a functional impairment of cholinergic transmission in the entorhinal cortex following ovariectomy. The acetylcholinesterase inhibitor eserine reduces the amplitude of fEPSPs, by prolonging the effects of acetylcholine which causes an M_1-mediated reduction in glutamate release (Barrett & Chapman, 2013; Hamam et al., 2007) that is likely to affect both AMPA and NMDA receptor-mediated responses. Eserine had a reduced effect on field EPSPs in the lateral entorhinal cortex

in slices from ovariectomized animals. Ovariectomy is also likely to have similar effects in the medial entorhinal cortex which also showed reductions in AChE and M_1 receptor protein. The reduced effect of eserine could result from a reduction in endogenous release of acetylcholine, leading to smaller eserine-induced increases in acetylcholine concentration. Results are also consistent with the reduction in M_1 receptors that we have observed, which may include reductions in M_1 receptors on glutamate terminals.

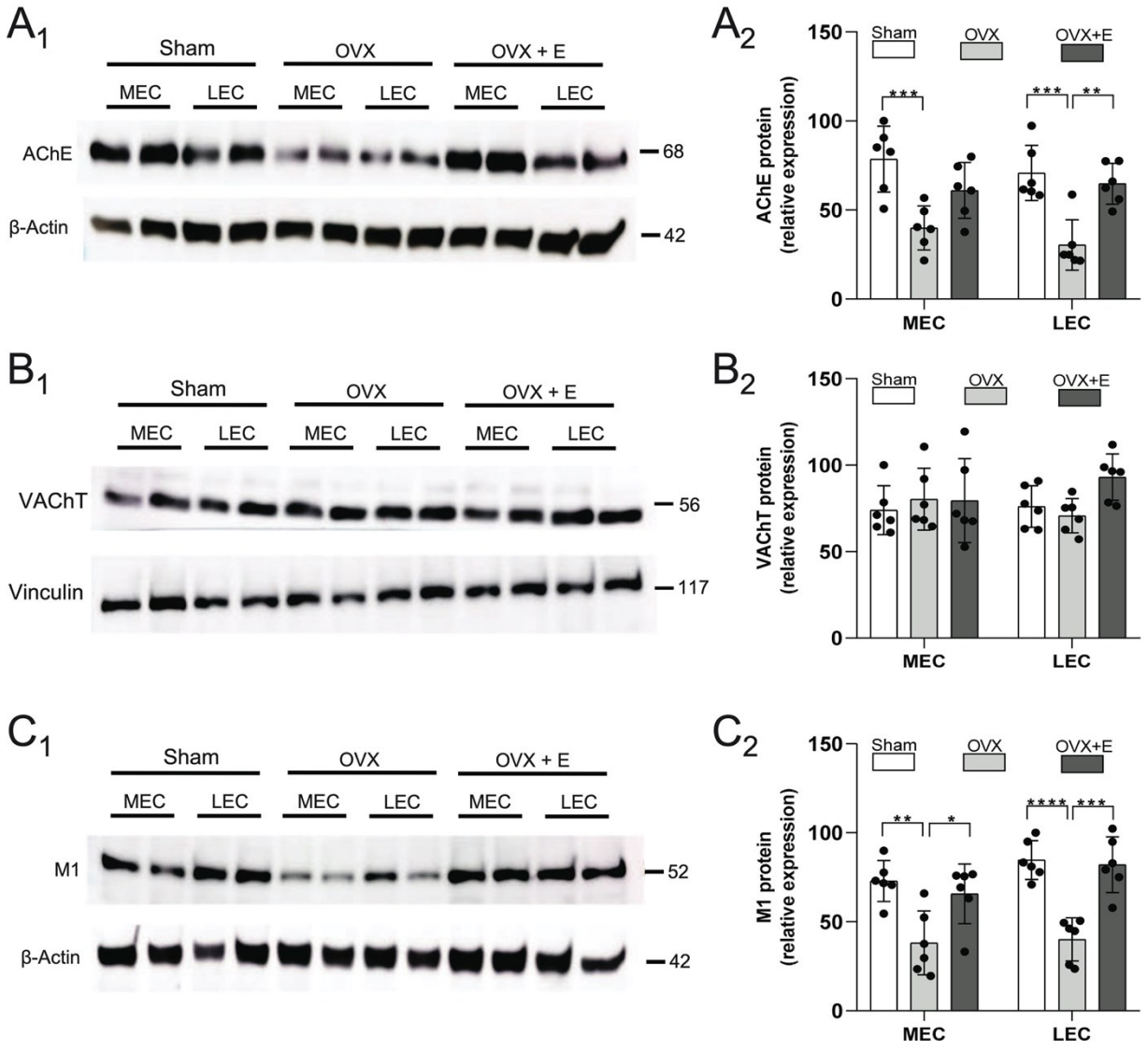
A1
Sham
OVX
OVX + E
MEC
LEC
MEC
LEC
MEC
LEC
AChE
β-Actin
68
42
A2
Sham
OVX
OVX+E
AChE protein (relative expression)
150
100
50
0
***
***
**
MEC
LEC
B1
Sham
OVX
OVX + E
MEC
LEC
MEC
LEC
MEC
LEC
VAChT
Vinculin
56
117
B2
Sham
OVX
OVX+E
VAChT protein (relative expression)
150
100
50
0
MEC
LEC
C1
Sham
OVX
OVX + E
MEC
LEC
MEC
LEC
MEC
LEC
M1
β-Actin
52
42
C2
Sham
OVX
OVX+E
M1 protein (relative expression)
150
100
50
0
**
*
****
***
MEC
LEC

Figure 4.1. Ovariectomy results in reductions in proteins associated with cholinergic synaptic function in the entorhinal cortex. Lysates were obtained from the medial and lateral entorhinal cortex (MEC and LEC), in groups of animals that received either sham surgery (Sham), ovariectomy (OVX), or ovariectomy and a subdermal implant containing 17-β estradiol (E2; OVX+E). **A.** Representative immunoblots of acetylcholinesterase (AChE) and the β-actin loading control are shown (A_1), and the bar graph shows relative expression of AChE protein (A_2; n=6 per group). Note that the reduction in AChE induced by ovariectomy is prevented by administration of E2. Asterisks indicate levels of statistical significance (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$; ****, $p < 0.0001$). **B.** No significant changes were observed in immunoblots (B_1) or normalized protein expression (B_2) for the vesicular acetylcholine transporter (VAChT; n=6 per group; vinculin was the loading control). **C.** Representative immunoblots (C_1) and relative protein expression (C_2) indicate that the reduction in M1 receptor protein induced by ovariectomy was prevented by administration of E2.

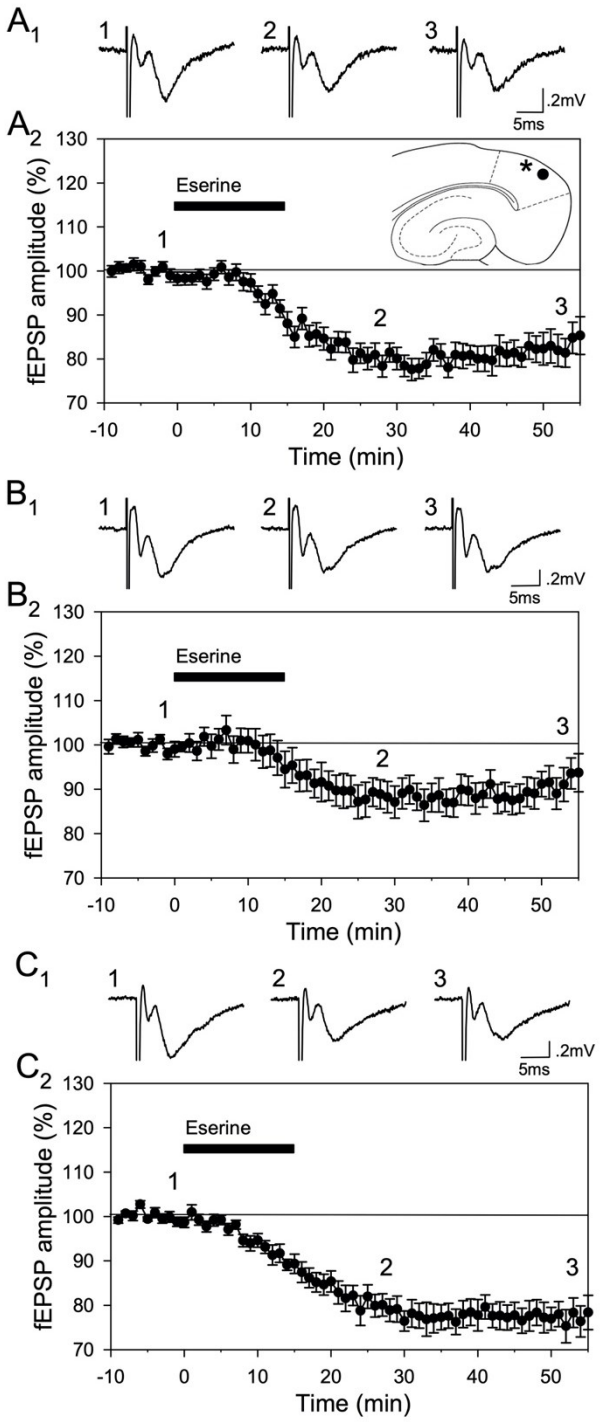
A1
1
2
3
.2mV
5ms
A2
Eserine
fEPSP amplitude (%)
Time (min)
B1
B2
C1
C2

Figure 4.2. Ovariectomy reduces cholinergic modulation of excitatory synapses. The acetylcholinesterase inhibitor eserine differentially modulates field excitatory postsynaptic potentials (fEPSPs) in the lateral entorhinal cortex in brain slices obtained from intact rats (A), ovariectomized rats (B), and ovariectomized rats with E2 capsules (C). A. Representative averaged fEPSPs from a slice obtained from an intact rat (A_1) are shown for the baseline period (trace 1), 28 min after onset of application of 10 μM eserine (trace 2), and at the end of the recording period (trace 3). Traces are averages of five consecutive responses. In slices from intact rats, mean fEPSP amplitude was reduced following application of eserine (black bar; n = 13) (A_2). The inset diagram shows typical locations of the stimulating (*) and recording (circle) electrodes in horizontal slices of the lateral entorhinal cortex. Numbers indicate the times at which traces in A1 were obtained, and bars indicate ± one standard error of the mean. B. Representative averaged traces are shown for a slice obtained from an ovariectomized rat (B_1), and changes in mean amplitude of fEPSPs in the group of slices are shown (B_2; n = 10). C. Representative traces (C_1) and averaged changes in mean fEPSP amplitude (C_2, n = 10) are shown for a group of slices obtained from ovariectomized rats that received E2 replacement. Reductions in fEPSP amplitude induced by eserine were significantly smaller in ovariectomized rats versus either intact rats or ovariectomized rats that received E2 replacement, both 25–30 min and 50–55 min after eserine application ($p < 0.05$).

CHAPTER 5

GENERAL DISCUSSION

Estrogens are primarily characterized as reproductive hormones, but they also contribute to cognitive processes including spatial navigation, object recognition and memory, and social learning (Hamson et al., 2016). The application of estrogen to the hippocampus can result in rapid increases in synaptic strength (Oberlander & Woolley, 2016), and loss of estrogens can result in cognitive disruptions associated with changes in the hippocampus and cholinergic systems (Hamson et al., 2016). The changes in cognitive function associated with estrogens have often been attributed to the modulatory effects of estrogen in the hippocampus. However, the entorhinal cortex contributes to cognitive and mnemonic functions of the medial temporal lobe, and and it is likely that modulation of synaptic function in the entorhinal cortex contributes to the cognitive effects of estrogens. This thesis explored the role of ovarian hormones, particularly estrogens, to the modulation and maintenance of synaptic function within the entorhinal cortex. Findings indicate that estrogens can have rapid effects on excitatory synaptic transmission in layers II/III of the entorhinal cortex through the activation of GPER1 receptors, and through a resulting inhibition of inhibitory synaptic transmission. Although there is a great deal of work exploring the effects of estrogens on the hippocampus (Kumar & Foster, 2020), this thesis has made a fundamental contribution to understanding how ovarian hormones affect the entorhinal cortex.

To better understand the contribution of ovarian hormones to synaptic function in entorhinal cortex, Chapter 2 explored the effect of ovarian hormones on excitatory transmission using electrically evoked excitatory postsynaptic field potentials (fEPSPs), a measure of synaptic transmission in layers I and II of the entorhinal cortex (Batallán Burrowes et al., 2021). Recordings consisted of a 10-min period of stable baseline recordings, followed by a 20-min exposure of 17β-estradiol (E2), progesterone, or allopregnanolone perfused through the bath, and a 30-min wash-off period to assess the reversibility of any effects. There was no effect of progesterone during the 20-min application period, but E2 did significantly increase the amplitude of fEPSPs, and this effect dissipated during the wash. To determine which estrogen receptors (ERs) contributed to this effect, selective ERα, ERβ, and GPER1 agonists were applied during the 20-min exposure period. Only application of the GPER1 agonist, G1, significantly increased fEPSPs similarly to E2, suggesting that activation of GPER1 receptors drives the facilitation of fEPSPs and excitatory synaptic transmission. This was further supported by the inability of E2 to facilitate fEPSPs in the presence of the GPER1 antagonist G15. These data

were the first to demonstrate the effects of ovarian hormones on excitatory synaptic transmission in the entorhinal cortex.

The findings from Chapter 2 demonstrated that estrogen induces a moderate increase in excitatory synaptic strength that is mediated by GPER1 receptors, but it did not demonstrate how this effect is expressed at excitatory synapses. The stability of the paired-pulse ratio before and during application of estrogen suggested that the increase in synaptic strength was expressed by an increased postsynaptic responsiveness, as opposed to an increased presynaptic release of glutamate. Postsynaptically, excitatory synaptic strength can be increased through increased activation of AMPA and/or NMDA glutamate receptors, but it could also result from a reduction in inhibitory synaptic transmission. In the hippocampus, previous research has shown that estrogen can have rapid effects on AMPA receptors (M. M. Khan et al., 2013), NMDA receptors (Tanaka & Sokabe, 2013), and can also suppress inhibitory transmission (Tabatadze et al., 2015). Importantly, Woolley and colleagues (G. Z. Huang & Woolley, 2012; Smejkalova & Woolley, 2010; Tabatadze et al., 2015) have found reduced inhibitory synaptic transmission can result in an increase in postsynaptic excitability. Although increased levels of E2 facilitated excitatory synaptic transmission in the entorhinal cortex via activation of GPER1 in Chaper 2, it was not clear how postsynaptic GPER1 modulated synaptic mechanisms to facilitate fEPSP.

Chapter 3 explored the effects of E2 on NMDA- and AMPA-mediated excitatory postsynaptic currents (EPSCs) and $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs) in entorhinal cortex principal neurons using whole-cell patch-clamp recordings. Similar to Chapter 2, following stable baseline recordings, E2 was perfused for a 20-min period, followed by a 20-min wash. Results showed no significant change to isolated AMPA-mediated EPSCs during E2 exposure, but did show significantly reduced NMDA-mediated EPCSs and $GABA_A$-mediated IPSCs. The change in IPSCs reversed towards baseline levels during the wash; however, NMDA-mediated EPSCs, did not show any signs of reversing, indicating a more persistent decrease in EPCSs. These findings therefore strongly suggest that activation of GPER1 receptors results in the facilitation of synaptic strength in layer II/III of the entorhinal cortex through a reduction in synaptic inhibition. These results provide insight into the mechanisms through which estrogens may modulate cognition in the entorhinal cortex.

The final experiments in this thesis examined the long-term effects of the loss of estrogens in ovariectomized female rats. The loss of estrogens is believed to contribute to

cognitive decline as a result of dysregulation of neuromodulatory transmitter systems (Ch'ng et al., 2020; Hwang et al., 2020), including the cholinergic system (Hammond & Gibbs, 2011). The loss of estrogens has been linked to a dysregulation and impairment of cholinergic function in various regions, including the hippocampus (Hammond et al., 2011) Gibbs and colleagues have shown how prolonged loss of estrogens following ovariectomy reduces the density of cholinergic neurons and projections from the basal forebrain and impairs cholinergic transmission to the hippocampus (Gibbs, 1998; Gibbs et al., 2014; Hammond et al., 2009, 2011). Cholinergic transmission is important for cognitive and mnemonic function within the hippocampus and entorhinal cortex (Hasselmo, 2006). The loss of cholinergic inputs to the entorhinal cortex could alter excitatory synaptic function and interfere with the cognitive processes mediated by the entorhinal cortex. However, the effects of the prolonged loss of estrogens on synaptic and cholinergic function in the entorhinal cortex were not known.

Chapter 4 examined the effects of ovariectomy on proteins associated with cholinergic function and its impact on the cholinergic modulation of excitatory synaptic responses (Batallán Burrowes et al., 2022). Western blots were used to measure differences in concentrations of acetylcholinesterase (AChE), vesicular acetylcholine transporter (vAChT), and M_1 receptor protein in groups that received ovariectomywith and without E2 replacement, and in intact female rats. Rats that underwent ovariectomywithout E2 replacement had significantly lower concentrations of AChE and M_1 in comparison to intact females and ovariectomy animals receiving E2 replacement, although vAChT did not change. This supports the idea that prolonged loss of estrogens significantly impairs cholinergic function in the entorhinal cortex. To assess the functional impact of changes in cholinergic transmission, changes in fEPSPs were measured during application of eserine (physostigmine), a AChE inhibitor. Because AChE degrades acetylcholine released in the synapse, the inhibition of AChE by eserine prolongs the synaptic presence of acetylcholine and increases its activity (Mans et al., 2014). Previous work has shown that increased acetylcholine activity supresses the release of glutamate via activation of presynaptic M_1 recptors and reduces excitatory synaptic transmission (Barrett & Chapman, 2013). Accordingly, recordings collected from all rats exhibited a significant decrease in fEPSP amplitude following application of eserine. However, intact rats and ovariectomy rats with E2 replacement showed significantly greater reductions in fEPSPs as compared to rats that received ovariectomy only. The disparity in cholinergic modulation of fEPSPs suggests that cholinergic

function is reduced in rats following ovariectomy and that E2 supplementation may prevent or decrease the severity of this alteration or impairment.

Loss of estrogens and menopause

Results of Chapter 4 point to important estrogen-dependent alterations in synaptic function in the entorhinal cortex following ovariectomy. Although this is likely to contribute to cognitive alterations associated with loss of estrogens in an important way, there are multiple neurophysiological alterations that occur throughout the brain following reductions in ovarian hormones. The primary neurophysiological mechanisms through which natural and surgical loss of estrogens affect cognitive and synaptic function throughout the brain are still not entirely clear. Clinical studies of cognitive function in premenopausal individuals transitioning to menopause and postmenopausal individuals report inconsistent results, with some reporting a significant change, transient change, or no change (Newhouse & Dumas, 2015). This inconsistency may be due to individual variations in genetics, medical history, parity, and lifestyle, that are not always considered in each study (Engler-Chiurazzi et al., 2017). Animal models also demonstrate a degree of inconsistency which can be attributed to subtle differences in methodology or individual differences in the type of animal, sex, and strain used (Hadjimarkou & Vasudevan, 2018; Hara et al., 2015).

In the basal forebrain, work by Gibbs and colleagues demonstrated how cholinergic function is modulated by the estrous cycle (Gibbs 1996) and provides significant benefit in the maintenance of cholinergic neuron function and projections (Hammond et al., 2009, 2011; Kompoliti et al., 2004). The loss of estrogens following ovariectomy results in significant decreases in synaptic spine densities in the hippocampus, reduced cholinergic cell bodies in the basal forebrain, and disrupts cholinergic transmissions and projections from the basal forebrain to the hippocampus (Hammond et al., 2011). There are also decreases in the concentration of BDNF (Kiss et al., 2012), reduced estrogen receptor expression and density (J. Wang et al., 2018), impaired mitochondrial function (W. Zhao et al., 2021), and increases in proinflammatory cytokines (T. Zhao et al., 2016). Supplementation of estrogens in animal models has been shown to prevent or rescue cognitive impairments and synaptic dysfunction induced by ovariectomy (Hammond & Gibbs, 2011). Gibbs et al. (2014) have attributed the loss of cognitive function in part to loss of basal forebrain cholinergic neurons, and a resulting loss of cholinergic inputs to

the hippocampal region (Hammond et al., 2011). The present results suggest these effects may be partly due to disruption of cholinergic inputs to the entorhinal cortex.

Impact of estrogens in the entorhinal cortex on cognitive function

The entorhinal cortex is a site of convergence of inputs from primary and secondary cortical areas. It receives strong inputs from the primary olfactory (piriform) cortex, perirhinal, and postrhinal cortices, which carry visual, auditory and associational inputs (Nilssen et al., 2019). Modulation of synaptic transmission in the entorhinal cortex may therefore affect the strength or salience of cortical inputs to the entorhinal cortex, and may also affect the manner in which this information is integrated. The entorhinal cortex is also well known to provide the largest cortical input to the hippocampus (Nilssen et al., 2019). Chapter 2 of this thesis determined that 17β-estradiol rapidly facilitates synaptic responses in layer II/III entorhinal neurons (Batallán Burrowes et al., 2021). Layer II neurons project via the perforant path to the dentate gyrus of the hippocampus, and layer III entorhinal neurons project to hippocampal CA3 and CA1 regions via the temperoammonic pathway (Burwell, 2000). Increases in the synaptic activation of entorhinal neurons that project from the entorhinal cortex to the hippocampus may therefore affect the manner in which integrated representations in the entorhinal cortex are projected to the hippocampus. This could increase the salience of these representations in the hippocampus and affect the manner in which they are processed and remembered.

Results obtained from both rats and people demonstrate shifts in cognitive strategy dependent on the reproductive cycle phase and the concentration of circulating estrogens. Work by Korol and colleagues (2004) shows how the estrous cycle can influence the preferred cognitive strategies rats use to solve the elevated plus maze, with proestrus and high estrogens promoting place-based strategies, while estrus promoted the use of response-based strategies. Similar shifts are observed in ovariectomized rats receiving control (low E) or replacement (high E) injections (Korol & Kolo, 2002). The changes in strategies associated with estrogen in rats suggests that increased estrogen promotes the use of strategies that are dependent on the hippocampal formation. This is consistent with the enhancing effects of estrogen on synaptic excitability in the hippocampus (Kumar et al., 2015; Oberlander & Woolley, 2016). In addition, however, the entorhinal cortex is thought to be strongly involved in spatial memory and navigation through the contribution of grid cells in the entorhinal cortex (Knierim et al., 2013). The results presented in this thesis show that estrogen can also enhance synaptic excitability in

the entorhinal cortex. This suggests that the shift in cognitive strategies to the use of spatial strategies during periods of higher levels of estrogen may to some degree involve the enhancement of excitatory synaptic responses in the entorhinal cortex.

Future Directions

Chapters 2 and 3 of this thesis demonstrated faciliatory effects of estrogens on synaptic transmission in the entorhinal cortex through activation of membrane-bound GPER1 receptors, and through a suppression of inhibitory transmission. The intracellular signalling mechanisms through which the activation of GPER1 receptors leads to changes in inhibitory synaptic transmission, however, are not yet clear. This could involve increased release of calcium from internal stores in the endoplasmic reticulum (Waters et al., 2015), modulation of other second messenger cascades including ERK and endocannabinoid signalling (Tabatadze et al., 2015), and/or the modulation of synaptic signalling mechanisms like neurotransmitter packaging, release, metabolism, or receptor activity (Oberlander & Woolley, 2016). GPER1-induced alteration of inhibitory synaptic transmission is not the only mechanism through which estrogens may facilitate excitatory synaptic transmission. Whole-cell recording experiments are needed to assess the contributions of alternative mechanisms through which estrogens may rapidly facilitate synaptic transmission. Given the relatively high expression of ERβ receptors in the entorhinal cortex and their probable colocalization with interneurons subtypes (Kritzer, 2002; Waters et al., 2015), it is also of interest to understand how estrogens may affect inhibitory interneuron networks through activation of ERβ receptors. This could be approached using a combination of selective ERβ agonists labeling of parvalbumin positive interneurons with a fluorescing protein to allow for the selective targeting of parvalbumin interneurons that may expresss ERβ for electrophysiological recordings in layers V and VI of the entorhinal cortex (Fernandez et al., 2022; Kritzer, 2002; Pisansky et al., 2019; Witter et al., 2017).

It is also important to confirm if the findings presented in chapters 2 and 3 also translate to the medial entorhinal cortex. All of the work presented here was conducted exclusively within the lateral entorhinal cortex, and suggests that estrogen may enhance functions of the lateral entorhinal cortex such as object recognition and olfaction (Knierim et al., 2013). However, given the distribution of GPER1 receptors in both the medial and lateral entorhinal cortex (Waters et

al., 2015) it is likely that similar effects on synaptic transmission could also occur in the medial entorhinal cortex and affect spatial navigation and mapping (Nilssen et al., 2019).

The entorhinal cortex is an integral part of the hippocampal region (Nilssen et al., 2019), and it is also important to understand how the effects of estrogen in the entorhinal cortex may impact hippocampal function. Neurons in the superficial layers of the entorhinal cortex project to the dentate gyrus and hippocampus, and it is therefore likely that effects of estrogens in the entorhinal cortex may result in increased excitability in the hippocampus. Experiments could be conducted *ex vivo,* using multi-electrode arrays to record changes in network activity induced by estrogen across the entorhinal and hippocampal regions (Becchetti et al., 2012; Kim et al., 2006). If estrogen-dependent increases in the activity of entorhinal neurons impact the activity of hippocampal neurons, this may be a way in which the coding of spatial and environmental stimuli within the entorhinal cortex may contribute to improved hippocampal-dependent cognitive function.

To further elucidate the role of ovarian hormones on synaptic function in the entorhinal cortex, further experiments are required to assess the effects progestogens in the entorhinal cortex. Although the female rats used in Chapter 2 were primed with E2 injections to increase progesterone receptor densities prior to recording (Guerra-Araiza et al., 2002; Micevych & Sinchak, 2008; Parsons et al., 1980), there were no effects of progesterone on fEPSPs in the entorhinal cortex. It is possible that progesterone does not have rapid effects on neuronal function in the entorhinal cortex, but rather induces slower genomic effects on entorhinal neurons. Future studies should explore the use of whole-cell recordings to better detect possible rapid effects of progesterone on synaptic potentials and neuronal excitability (Edwards et al., 2000). Further work could also be done to study the effects of allopregnanolone, which is a major a metabolite of progesterone. The effects of allopregnanolone are not dependent upon progesterone receptors as allopregnanolone can directly enhance inhibition via activation of ionotropic GABA receptors (González-Orozco & Camacho-Arroyo, 2019; Nin et al., 2011; Wang, 2011). Whole-cell recordings of IPSCs in the presence of allopregnanolone could assess possible changes inhibitory synaptic transmission that may occur in individual cells.
Changes in the concentration of estrogens and cholinergic function associated with ovariectomy may have important implications for the neuropathology of Alzheimer's Disease (AD). AD is more common in women than in men, and estrogens are thought to provide some

neuroprotection against the development and progression of AD, while loss of estrogens may increase susceptibility to AD in woman after menopause (Hamson et al., 2016). Early signs of AD neuropathology have been reported in the entorhinal cortex (Khan et al., 2014), and understanding how estrogens may protect against mechanisms of AD in the entorhinal cortex can inform our understanding of AD development and treatment. Furthermore, estrogens promote cholinergic function within the basal forebrain where cholinergic neurons are located, and within cholinergic projection areas in the medial temporal lobe (Hammond & Gibbs, 2011; Kondo & Zaborszky, 2016). The loss of estrogens following menopause may therefore impair cholinergic function and may contribute to the symptomatology of AD (Newhouse & Dumas, 2015). These factors, combined with the accumulation of amyloid β protein (Aβ) in the entorhinal cortex, likely contribute to the neurodegeneration and cognitive impairment that females experience early in AD (Hamson et al., 2016). However, further study of the interactions between cholinergic function, estrogens, and Aβ in the entorhinal cortex is necessary to better understand the development of early AD pathology. Of particular interest is their impact on synaptic function and transmission, and how risk factors associated with individual and experiential differences, such as parity, may influence AD progression.

REFERENCES

Acconcia, F., & Marino, M. (2018). Principles of Endocrinology and Hormone Action. *Endocrinology*, 43–72. https://doi.org/10.1007/978-3-319-44675-2_4

Akama, K. T., Thompson, L. I., Milner, T. A., & McEwen, B. S. (2013). Post-synaptic Density-95 (PSD-95) Binding Capacity of G-protein-coupled Receptor 30 (GPR30), an Estrogen Receptor That Can Be Identified in Hippocampal Dendritic Spines. *Journal of Biological Chemistry*, *288*(9), 6438–6450. https://doi.org/10.1074/jbc.m112.412478

Alexander, A., Irving, A. J., & Harvey, J. (2017). Emerging roles for the novel estrogen-sensing receptor GPER1 in the CNS. *Neuropharmacology*, *113*, 652–660. https://doi.org/10.1016/j.neuropharm.2016.07.003

Almey, A., Cannell, E., Bertram, K., Filardo, E., Milner, T. A., & Brake, W. G. (2014). Medial Prefrontal Cortical Estradiol Rapidly Alters Memory System Bias in Female Rats: Ultrastructural Analysis Reveals Membrane-Associated Estrogen Receptors as Potential Mediators. *Endocrinology*, *155*(11), 4422–4432. https://doi.org/10.1210/en.2014-1463

Almey, A., Hafez, N. M., Hantson, A., & Brake, W. G. (2013). Deficits in latent inhibition induced by estradiol replacement are ameliorated by haloperidol treatment. *Frontiers in Behavioral Neuroscience*, *7*, 136. https://doi.org/10.3389/fnbeh.2013.00136

Almey, A., Milner, T. A., & Brake, W. G. (2015). Estrogen receptors in the central nervous system and their implication for dopamine-dependent cognition in females. *Hormones and Behavior*, *74*, 125–138. https://doi.org/10.1016/j.yhbeh.2015.06.010

Álvarez-Delgado, C., Mendoza-Rodríguez, C. A., Picazo, O., & Cerbón, M. (2010). Different expression of α and β mitochondrial estrogen receptors in the aging rat brain: Interaction with respiratory complex V. *Experimental Gerontology*, *45*(7–8), 580–585. https://doi.org/10.1016/j.exger.2010.01.015

Arevalo, M.-A., Azcoitia, I., Gonzalez-Burgos, I., & Garcia-Segura, L. M. (2015). Signaling mechanisms mediating the regulation of synaptic plasticity and memory by estradiol. *Hormones and Behavior*, *74*, 19–27. https://doi.org/10.1016/j.yhbeh.2015.04.016

Barbieri, R. L. (2014). Human Fertility, Methods and Protocols. *Methods in Molecular Biology*, *1154*, 145–169. https://doi.org/10.1007/978-1-4939-0659-8_7

Barrett, S. G., & Chapman, A. C. (2013). Contribution of muscarinic M1 receptors to the cholinergic suppression of synaptic responses in layer II of the entorhinal cortex. *Neuroscience Letters*, *554*, 11–15. https://doi.org/10.1016/j.neulet.2013.08.050

Barros, L. A., Tufik, S., & Andersen, M. L. (2015). The role of progesterone in memory: An overview of three decades. *Neuroscience & Biobehavioral Reviews*, *49*, 193–204. https://doi.org/10.1016/j.neubiorev.2014.11.015

Batallán Burrowes, A. A., Olajide, O. J., Iasenza, I. A., Shams, W. M., Carter, F., & Chapman, C. A. (2022). Ovariectomy reduces cholinergic modulation of excitatory synaptic transmission in the rat entorhinal cortex. *PLoS ONE*, *17*(8), e0271131. https://doi.org/10.1371/journal.pone.0271131

Batallán Burrowes, A. A., Sundarakrishnan, A., Bouhour, C., & Chapman, C. A. (2021). G protein-coupled estrogen receptor-1 enhances excitatory synaptic responses in the entorhinal cortex. *Hippocampus*, *31*(11), 1191–1201. https://doi.org/10.1002/hipo.23383

Bauzá-Thorbrügge, M., Rodríguez-Cuenca, S., Vidal-Puig, A., Galmés-Pascual, B. M., Sbert-Roig, M., Gianotti, M., Lladó, I., & Proenza, A. M. (2019). GPER and ERα mediate estradiol enhancement of mitochondrial function in inflamed adipocytes through a PKA dependent mechanism. *The Journal of Steroid Biochemistry and Molecular Biology*, *185*, 256–267. https://doi.org/10.1016/j.jsbmb.2018.09.013

Bean, L. A., Ianov, L., & Foster, T. C. (2014). Estrogen Receptors, the Hippocampus, and Memory. *The Neuroscientist*, *20*(5), 534–545. https://doi.org/10.1177/1073858413519865

Becchetti, A., Gullo, F., Bruno, G., Dossi, E., Lecchi, M., & Wanke, E. (2012). Exact distinction of excitatory and inhibitory neurons in neural networks: a study with GFP-GAD67 neurons optically and electrophysiologically recognized on multielectrode arrays. *Frontiers in Neural Circuits*, *6*, 63. https://doi.org/10.3389/fncir.2012.00063

Benmansour, S., Adeniji, O. S., Privratsky, A. A., & Frazer, A. (2016). Effects of Long-Term Treatment with Estradiol and Estrogen Receptor Subtype Agonists on Serotonergic Function in Ovariectomized Rats. *Neuroendocrinology*, *103*(3–4), 269–281. https://doi.org/10.1159/000437268

Bennett, K. M., Hoelting, C., Martin, C. P., & Stoll, J. (2009). Estrogen Effects on High-Affinity Choline Uptake in Primary Cultures of Rat Basal Forebrain. *Neurochemical Research*, *34*(2), 205–214. https://doi.org/10.1007/s11064-008-9746-1

Bernal, A., & Paolieri, D. (2021). The Influence of Estradiol and Progesterone on Neurocognition During Three Phases of the Menstrual Cycle: Modulating Factors. *Behavioural Brain Research*, *417*, 113593. https://doi.org/10.1016/j.bbr.2021.113593

Bianchini, F., Verde, P., Colangeli, S., Boccia, M., Strollo, F., Guariglia, C., Bizzarro, G., & Piccardi, L. (2018). Effects of oral contraceptives and natural menstrual cycling on environmental learning. *BMC Women's Health*, *18*(1), 179. https://doi.org/10.1186/s12905-018-0671-4

Björnström, L., & Sjöberg, M. (2005). Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Molecular Endocrinology*, *19*(4), 833–842. https://doi.org/10.1210/me.2004-0486

Blurton-Jones, M., & Tuszynski, M. H. (2002). Estrogen receptor-beta colocalizes extensively with parvalbumin-labeled inhibitory neurons in the cortex, amygdala, basal forebrain, and hippocampal formation of intact and ovariectomized adult rats. *Journal of Comparative Neurology*, *452*(3), 276–287. https://doi.org/10.1002/cne.10393

Blurton-Jones, M., & Tuszynski, M. H. (2006). Estradiol-induced modulation of estrogen receptor-β and GABA within the adult neocortex: A potential transsynaptic mechanism for estrogen modulation of BDNF. *Journal of Comparative Neurology*, *499*(4), 603–612. https://doi.org/10.1002/cne.21122

Boyle, C. P., Raji, C. A., Erickson, K. I., Lopez, O. L., Becker, J. T., Gach, H. M., Kuller, L. H., Longstreth, W., Carmichael, O. T., Riedel, B. C., & Thompson, P. M. (2021). Estrogen, brain structure, and cognition in postmenopausal women. *Human Brain Mapping*, *42*(1), 24–35. https://doi.org/10.1002/hbm.25200

Brailoiu, E., Dun, S. L., Brailoiu, G. C., Mizuo, K., Sklar, L. A., Oprea, T. I., Prossnitz, E. R., & Dun, N. J. (2007). Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *Journal of Endocrinology*, *193*(2), 311–321. https://doi.org/10.1677/joe-07-0017

Brann, D. W., Lu, Y., Wang, J., Zhang, Q., Thakkar, R., Sareddy, G. R., Pratap, U. P., Tekmal, R. R., & Vadlamudi, R. K. (2022). Brain-derived estrogen and neural function. *Neuroscience and Biobehavioral Reviews*, *132*, 793–817. https://doi.org/10.1016/j.neubiorev.2021.11.014

Brinton, R. D. (2004). Impact of Estrogen Therapy on Alzheimer's Disease. *CNS Drugs*, *18*(7), 405–422. https://doi.org/10.2165/00023210-200418070-00001

Burwell, R. D. (2000). The Parahippocampal Region: Corticocortical Connectivity. *Annals of the New York Academy of Sciences*, *911*(1), 25–42. https://doi.org/10.1111/j.1749-6632.2000.tb06717.x

Cardoso, C. C., Ricardo, V. P., Frussa-Filho, R., Porto, C. S., & Abdalla, F. M. F. (2010). Effects of 17β-estradiol on expression of muscarinic acetylcholine receptor subtypes and estrogen receptor α in rat hippocampus. *European Journal of Pharmacology*, *634*(1–3), 192–200. https://doi.org/10.1016/j.ejphar.2010.02.032

Ch'ng, S. S., Walker, A. J., McCarthy, M., Le, T.-K., Thomas, N., Gibbons, A., Udawela, M., Kusljic, S., Dean, B., & Gogos, A. (2020). The Impact of Removal of Ovarian Hormones on Cholinergic Muscarinic Receptors: Examining Prepulse Inhibition and Receptor Binding. *Brain Sciences*, *10*(2), 106. https://doi.org/10.3390/brainsci10020106

Clemens, A. M., Lenschow, C., Beed, P., Li, L., Sammons, R., Naumann, R. K., Wang, H., Schmitz, D., & Brecht, M. (2019). Estrus-Cycle Regulation of Cortical Inhibition. *Current Biology*, *29*(4), 605-615.e6. https://doi.org/10.1016/j.cub.2019.01.045

Coelingh Bennink, H. J. T., Verhoeven, C., Zimmerman, Y., Visser, M., Foidart, J.-M., & Gemzell-Danielsson, K. (2016). Clinical effects of the fetal estrogen estetrol in a multiple-rising-dose study in postmenopausal women. *Maturitas*, *91*, 93–100. https://doi.org/10.1016/j.maturitas.2016.06.017

Cruz, G., Fernandois, D., & Paredes, A. H. (2017). Ovarian function and reproductive senescence in the rat: role of ovarian sympathetic innervation. *Reproduction*, *153*(2), R59–R68. https://doi.org/10.1530/rep-16-0117

Daniel, J. M., & Dohanich, G. P. (2001). Acetylcholine Mediates the Estrogen-Induced Increase in NMDA Receptor Binding in CA1 of the Hippocampus and the Associated Improvement in Working Memory. *Journal of Neuroscience*, *21*(17), 6949–6956. https://doi.org/10.1523/jneurosci.21-17-06949.2001

Daniel, J. M., Fader, A. J., Spencer, A. L., & Dohanich, G. P. (1997). Estrogen Enhances Performance of Female Rats during Acquisition of a Radial Arm Maze. *Hormones and Behavior*, *32*(3), 217–225. https://doi.org/10.1006/hbeh.1997.1433

Dannenberg, H., Hinman, J. R., & Hasselmo, M. E. (2016). Potential roles of cholinergic modulation in the neural coding of location and movement speed. *Journal of Physiology-Paris*, *110*(1–2), 52–64. https://doi.org/10.1016/j.jphysparis.2016.09.002

Day, M., Sung, A., Logue, S., Bowlby, M., & Arias, R. (2005). Beta estrogen receptor knockout (BERKO) mice present attenuated hippocampal CA1 long-term potentiation and related memory deficits in contextual fear conditioning. *Behavioural Brain Research*, *164*(1), 128–131. https://doi.org/10.1016/j.bbr.2005.05.011

DeFelipe, J. (1993). Neocortical Neuronal Diversity: Chemical Heterogeneity Revealed by Colocalization Studies of Classic Neurotransmitters, Neuropeptides, Calcium-binding Proteins, and Cell Surface Molecules. *Cerebral Cortex*, *3*(4), 273–289. https://doi.org/10.1093/cercor/3.4.273

DeFelipe, J. (1997). Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. *Journal of Chemical Neuroanatomy*, *14*(1), 1–19. https://doi.org/10.1016/s0891-0618(97)10013-8

Deshmukh, S. S., & Knierim, J. J. (2011). Representation of Non-Spatial and Spatial Information in the Lateral Entorhinal Cortex. *Frontiers in Behavioral Neuroscience*, *5*, 69. https://doi.org/10.3389/fnbeh.2011.00069

Domańska, A., Orzechowski, A., Litwiniuk, A., Kalisz, M., Bik, W., & Baranowska-Bik, A. (2021). The Beneficial Role of Natural Endocrine Disruptors: Phytoestrogens in Alzheimer's Disease. *Oxidative Medicine and Cellular Longevity*, *2021*, 3961445. https://doi.org/10.1155/2021/3961445

Doncheck, E. M., Anderson, E. M., Konrath, C. D., Liddiard, G. T., DeBaker, M. C., Urbanik, L. A., Hearing, M. C., & Mantsch, J. R. (2021). Estradiol Regulation of the Prelimbic Cortex and the Reinstatement of Cocaine Seeking in Female Rats. *The Journal of Neuroscience*, *41*(24), 5303–5314. https://doi.org/10.1523/jneurosci.3086-20.2021

Duarte-Guterman, P., Albert, A. Y., Barha, C. K., Galea, L. A. M., & Initiative, on behalf of the A. D. N. (2021). Sex influences the effects of APOE genotype and Alzheimer's diagnosis on neuropathology and memory. *Psychoneuroendocrinology*, *129*, 105248. https://doi.org/10.1016/j.psyneuen.2021.105248

Edwards, H. E., Epps, T., Carlen, P. L., & MacLusky, N. J. (2000). Progestin receptors mediate progesterone suppression of epileptiform activity in tetanized hippocampal slices in vitro. *Neuroscience*, *101*(4), 895–906. https://doi.org/10.1016/S0306-4522(00)00439-5

Eichenbaum, H., Yonelinas, A. P., & Ranganath, C. (2007). The Medial Temporal Lobe and Recognition Memory. *Annual Review of Neuroscience*, *30*(1), 123–152. https://doi.org/10.1146/annurev.neuro.30.051606.094328

El-Bakri, N. K., Adem, A., Suliman, I. A., Mulugeta, E., Karlsson, E., Lindgren, J. U., Winblad, B., & Islam, A. (2002). Estrogen and progesterone treatment: effects on muscarinic M4 receptor subtype in the rat brain. *Brain Research*, *948*(1–2), 131–137. https://doi.org/10.1016/s0006-8993(02)02962-1

Engler-Chiurazzi, E. B., Brown, C. M., Povroznik, J. M., & Simpkins, J. W. (2017). Estrogens as neuroprotectants: Estrogenic actions in the context of cognitive aging and brain injury. *Progress in Neurobiology*, *157*, 188–211. https://doi.org/10.1016/j.pneurobio.2015.12.008

Erli, F., Palmos, A. B., Raval, P., Mukherjee, J., Sellers, K. J., Gatford, N. J. F., Moss, S. J., Brandon, N. J., Penzes, P., & Srivastava, D. P. (2020). Estradiol reverses excitatory synapse loss in a cellular model of neuropsychiatric disorders. *Translational Psychiatry*, *10*(1), 16. https://doi.org/10.1038/s41398-020-0682-4

Ertman, N., Andreano, J. M., & Cahill, L. (2011). Progesterone at encoding predicts subsequent emotional memory. *Learning & Memory*, *18*(12), 759–763. https://doi.org/10.1101/lm.023267.111

Ervin, K. S. J., Lymer, J. M., Matta, R., Clipperton-Allen, A. E., Kavaliers, M., & Choleris, E. (2015). Estrogen involvement in social behavior in rodents: Rapid and long-term actions. *Hormones and Behavior*, *74*, 53–76. https://doi.org/10.1016/j.yhbeh.2015.05.023

Ervin, K. S. J., Mulvale, E., Gallagher, N., Roussel, V., & Choleris, E. (2015). Activation of the G protein-coupled estrogen receptor, but not estrogen receptor α or β, rapidly enhances social learning. *Psychoneuroendocrinology*, *58*(Learn. Mem. 8 2001), 51–66. https://doi.org/10.1016/j.psyneuen.2015.04.002

Espinosa, E., & Curtis, K. S. (2018). Increased locomotor activity in estrogen-treated ovariectomized rats is associated with nucleus accumbens dopamine and is not reduced by dietary sodium deprivation. *Integrative Zoology*, *13*(6), 783–794. https://doi.org/10.1111/1749-4877.12333

Evans, P. D. (2019). Rapid signalling responses via the G protein-coupled estrogen receptor, GPER, in a hippocampal cell line. *Steroids*, *152*, 108487. https://doi.org/10.1016/j.steroids.2019.108487

Fan, L., Zhao, Z., Orr, P. T., Chambers, C. H., Lewis, M. C., & Frick, K. M. (2010). Estradiol-Induced Object Memory Consolidation in Middle-Aged Female Mice Requires Dorsal Hippocampal Extracellular Signal-Regulated Kinase and Phosphatidylinositol 3-Kinase Activation. *The Journal of Neuroscience*, *30*(12), 4390–4400. https://doi.org/10.1523/jneurosci.4333-09.2010

Fernandez, F. R., Via, G., Canavier, C. C., & White, J. A. (2022). Kinetics and Connectivity Properties of Parvalbumin- and Somatostatin-Positive Inhibition in Layer 2/3 Medial Entorhinal Cortex. *ENeuro*, *9*(1), ENEURO.0441-21.2022. https://doi.org/10.1523/eneuro.0441-21.2022

Ferreira, J. A., Foley, A. M., & Brown, M. (2012). Sex hormones differentially influence voluntary running activity, food intake and body weight in aging female and male rats. *European Journal of Applied Physiology*, *112*(8), 3007–3018. https://doi.org/10.1007/s00421-011-2271-y

Finney, C. A., Proschogo, N. W., Snepvangers, A., Holmes, N. M., Westbrook, R. F., & Clemens, K. J. (2020). The effect of standard laboratory diets on estrogen signaling and spatial memory in male and female rats. *Physiology & Behavior*, *215*, 112787. https://doi.org/10.1016/j.physbeh.2019.112787

Finney, C. A., Shvetcov, A., Westbrook, R. F., Jones, N. M., & Morris, M. J. (2020). The role of hippocampal estradiol in synaptic plasticity and memory: A systematic review. *Frontiers in Neuroendocrinology*, *56*, 100818. https://doi.org/10.1016/j.yfrne.2019.100818

Foy, M. R., Xu, J., Xie, X., Brinton, R. D., Thompson, R. F., & Berger, T. W. (1999). 17β-Estradiol Enhances NMDA Receptor-Mediated EPSPs and Long-Term Potentiation. *Journal of Neurophysiology*, *81*(2), 925–929. https://doi.org/10.1152/jn.1999.81.2.925

Frick, K. M., & Kim, J. (2018). Mechanisms underlying the rapid effects of estradiol and progesterone on hippocampal memory consolidation in female rodents. *Hormones and Behavior*, *104*, 100–110. https://doi.org/10.1016/j.yhbeh.2018.04.013

Gabor, C., Lymer, J., Phan, A., & Choleris, E. (2015). Rapid effects of the G-protein coupled oestrogen receptor (GPER) on learning and dorsal hippocampus dendritic spines in female mice. *Physiology & Behavior*, *149*, 53–60. https://doi.org/10.1016/j.physbeh.2015.05.017

Galea, L. A. M., Leuner, B., & Slattery, D. A. (2014). Hippocampal Plasticity during the Peripartum Period: Influence of Sex Steroids, Stress and Ageing. *Journal of Neuroendocrinology*, *26*(10), 641–648. https://doi.org/10.1111/jne.12177

Galea, L. A., Kavaliers, M., & Ossenkopp, K. P. (1996). Sexually dimorphic spatial learning in meadow voles Microtus pennsylvanicus and deer mice Peromyscus maniculatus. *Journal of Experimental Biology*, *199*(1), 195–200. https://doi.org/10.1242/jeb.199.1.195

Garcia, N., Walker, R., & Zoellner, L. (2018). Estrogen, progesterone, and the menstrual cycle: A systematic review of fear learning, intrusive memories, and PTSD. *Clinical Psychology Review*, *Biological Psychiatry 43 8 1998*. https://doi.org/10.1016/j.cpr.2018.06.005

Gervais, N. J., Jacob, S., Brake, W. G., & Mumby, D. G. (2013). Systemic and intra-rhinal-cortical 17-β estradiol administration modulate object-recognition memory in ovariectomized female rats. *Hormones and Behavior*, *64*(4), 642–652. https://doi.org/10.1016/j.yhbeh.2013.08.010

Gibbs, R. (1996). Fluctuations in relative levels of choline acetyltransferase mRNA in different regions of the rat basal forebrain across the estrous cycle: effects of estrogen and progesterone. *Journal of Neuroscience*, *16*(3), 1049–1055. https://doi.org/10.1523/jneurosci.16-03-01049.1996

Gibbs, R. B. (1997). Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. *Brain Research*, *757*(1), 10–16. https://doi.org/10.1016/s0006-8993(96)01432-1

Gibbs, R. B. (1998). Impairment of Basal Forebrain Cholinergic Neurons Associated with Aging and Long-Term Loss of Ovarian Function. *Experimental Neurology*, *151*(2), 289–302. https://doi.org/10.1006/exnr.1998.6789

Gibbs, R. B. (1999). Estrogen Replacement Enhances Acquisition of a Spatial Memory Task and Reduces Deficits Associated with Hippocampal Muscarinic Receptor Inhibition. *Hormones and Behavior*, *36*(3), 222–233. https://doi.org/10.1006/hbeh.1999.1541

Gibbs, R. B. (2000). Effects of gonadal hormone replacement on measures of basal forebrain cholinergic function. *Neuroscience*, *101*(4), 931–938. https://doi.org/10.1016/s0306-4522(00)00433-4

Gibbs, R. B. (2010). Estrogen Therapy and Cognition: A Review of the Cholinergic Hypothesis. *Endocrine Reviews*, *31*(2), 224–253. https://doi.org/10.1210/er.2009-0036

Gibbs, R. B., Nelson, D., & Hammond, R. (2014). Role of GPR30 in mediating estradiol effects on acetylcholine release in the hippocampus. *Hormones and Behavior*, *66*(2), 339–345. https://doi.org/10.1016/j.yhbeh.2014.06.002

Gibbs, R. B., Wu, D., Hersh, L. B., & Pfaff, D. W. (1994). Effects of Estrogen Replacement on the Relative Levels of Choline Acetyltransferase, trkA, and Nerve Growth Factor Messenger RNAs in the Basal Forebrain and Hippocampal Formation of Adult Rats. *Experimental Neurology*, *129*(1), 70–80. https://doi.org/10.1006/exnr.1994.1148

Gingerich, S., Kim, G. L., Chalmers, J. A., Koletar, M. M., Wang, X., Wang, Y., & Belsham, D. D. (2010). Estrogen receptor alpha and G-protein coupled receptor 30 mediate the neuroprotective effects of 17β-estradiol in novel murine hippocampal cell models. *Neuroscience*, *170*(1), 54–66. https://doi.org/10.1016/j.neuroscience.2010.06.076

Goethem, N. P. van, Rutten, K., Staay, F. J. van der, Jans, L. A. W., Akkerman, S., Steinbusch, H. W. M., Blokland, A., Klooster, J. van't, & Prickaerts, J. (2012). Object recognition testing: Rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research*, *232*(2), 323–334. https://doi.org/10.1016/j.bbr.2012.03.023

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *80*(2), 84–97. https://doi.org/10.1002/bdrb.20106

González-Orozco, J. C., & Camacho-Arroyo, I. (2019). Progesterone Actions During Central Nervous System Development. *Frontiers in Neuroscience*, *13*, 503. https://doi.org/10.3389/fnins.2019.00503

Gould, E., Woolley, C. S., Frankfurt, M., & McEwen, B. S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *Journal of Neuroscience*, *10*(4), 1286–1291. https://doi.org/10.1523/jneurosci.10-04-01286.1990

Graham, B. M., & Scott, E. (2018). Effects of systemic estradiol on fear extinction in female rats are dependent on interactions between dose, estrous phase, and endogenous estradiol levels. *Hormones and Behavior*, *97*, 67–74. https://doi.org/10.1016/j.yhbeh.2017.10.009

Guennoun, R. (2020). Progesterone in the Brain: Hormone, Neurosteroid and Neuroprotectant. *International Journal of Molecular Sciences*, *21*(15), 5271. https://doi.org/10.3390/ijms21155271

Guerra-Araiza, C., Coyoy-Salgado, A., & Camacho-Arroyo, I. (2002). Sex differences in the regulation of progesterone receptor isoforms expression in the rat brain. *Brain Research Bulletin*, *59*(2), 105–109. https://doi.org/10.1016/s0361-9230(02)00845-6

Hadjimarkou, M. M., & Vasudevan, N. (2018). GPER1/GPR30 in the brain: Crosstalk with classical estrogen receptors and implications for behavior. *The Journal of Steroid Biochemistry and Molecular Biology*, *176*, 57–64. https://doi.org/10.1016/j.jsbmb.2017.04.012

Hafting, T., Fyhn, M., Bonnevie, T., Moser, M.-B., & Moser, E. I. (2008). Hippocampus-independent phase precession in entorhinal grid cells. *Nature*, *453*(7199), 1248. https://doi.org/10.1038/nature06957

Hafting, T., Fyhn, M., Molden, S., Moser, M.-B., & Moser, E. I. (2005). Microstructure of a spatial map in the entorhinal cortex. *Nature*, *436*(7052), nature03721. https://doi.org/10.1038/nature03721

Hales, J. B., Vincze, J. L., Reitz, N. T., Ocampo, A. C., Leutgeb, S., & Clark, R. E. (2018). Recent and remote retrograde memory deficit in rats with medial entorhinal cortex lesions. *Neurobiology of Learning and Memory*, *155*, 157–163. https://doi.org/10.1016/j.nlm.2018.07.013

Hamam, B. N., Sinai, M., Poirier, G., & Chapman, C. A. (2007). Cholinergic suppression of excitatory synaptic responses in layer II of the medial entorhinal cortex. *Hippocampus*, *17*(2), 103–113. https://doi.org/10.1002/hipo.20249

Hammond, R., & Gibbs, R. B. (2011). GPR30 is positioned to mediate estrogen effects on basal forebrain cholinergic neurons and cognitive performance. *Brain Research*, *1379*, 53–60. https://doi.org/10.1016/j.brainres.2010.11.098

Hammond, R., Mauk, R., Ninaci, D., Nelson, D., & Gibbs, R. B. (2009). Chronic treatment with estrogen receptor agonists restores acquisition of a spatial learning task in young ovariectomized rats. *Hormones and Behavior*, *56*(3), 309–314. https://doi.org/10.1016/j.yhbeh.2009.06.008

Hammond, R., Nelson, D., & Gibbs, R. B. (2011). GPR30 co-localizes with cholinergic neurons in the basal forebrain and enhances potassium-stimulated acetylcholine release in the hippocampus. *Psychoneuroendocrinology*, *36*(2), 182–192. https://doi.org/10.1016/j.psyneuen.2010.07.007

Hamson, D. K., Roes, M. M., & Galea, L. A. M. (2016). Comprehensive Physiology. *Comprehensive Physiology*, *6*(3), 1295–1337. https://doi.org/10.1002/cphy.c150031

Hara, Y., Waters, E. M., McEwen, B. S., & Morrison, J. H. (2015). Estrogen Effects on Cognitive and Synaptic Health Over the Lifecourse. *Physiological Reviews*, *95*(3), 785–807. https://doi.org/10.1152/physrev.00036.2014

Harman, S. M., Brinton, E. A., Clarkson, T., Heward, C. B., Hecht, H. S., Karas, R. H., Judelson, D. R., & Naftolin, F. (2004). Is the WHI relevant to HRT started in the perimenopause? *Endocrine*, *24*(3), 195–202. https://doi.org/10.1385/endo:24:3:195

Hasselmo, M. E. (2006). The role of acetylcholine in learning and memory. *Current Opinion in Neurobiology*, *16*(6), 710–715. https://doi.org/10.1016/j.conb.2006.09.002

Hausmann, M., Slabbekoorn, D., Goozen, S. H. M. V., Cohen-Kettenis, P. T., & Güntürkün, O. (2000). Sex Hormones Affect Spatial Abilities During the Menstrual Cycle. *Behavioral Neuroscience*, *114*(6), 1245–1250. https://doi.org/10.1037/0735-7044.114.6.1245

Hazell, G. G. J., Yao, S. T., Roper, J. A., Prossnitz, E. R., O'Carroll, A.-M., & Lolait, S. J. (2009). Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. *Journal of Endocrinology*, *202*(2), 223–236. https://doi.org/10.1677/JOE-09-0066

Heys, J. G., Schultheiss, N. W., Shay, C. F., Tsuno, Y., & Hasselmo, M. E. (2012). Effects of acetylcholine on neuronal properties in entorhinal cortex. *Frontiers in Behavioral Neuroscience*, *6*, 32. https://doi.org/10.3389/fnbeh.2012.00032

Hilakivi-Clarke, L., Assis, S. de, & Warri, A. (2013). Exposures to Synthetic Estrogens at Different Times During the Life, and Their Effect on Breast Cancer Risk. *Journal of Mammary Gland Biology and Neoplasia*, *18*(1), 25–42. https://doi.org/10.1007/s10911-013-9274-8

Holden, C. (2010). Behavioral Addictions Debut in Proposed DSM-V. *Science*, *327*(5968), 935–935. https://doi.org/10.1126/science.327.5968.935

Horvath, K. M., Hårtig, W., Veen, R. der V., Keijser, J. N., Mulder, J., Ziegert, M., Zee, E. A. der V., Harkany, T., & Luiten, P. G. M. (2002). 17β-Estradiol enhances cortical cholinergic innervation and preserves synaptic density following excitotoxic lesions to the rat nucleus basalis magnocellularis. *Neuroscience*, *110*(3), 489–504. https://doi.org/10.1016/s0306-4522(01)00560-7

Huang, G. Z., & Woolley, C. S. (2012). Estradiol Acutely Suppresses Inhibition in the Hippocampus through a Sex-Specific Endocannabinoid and mGluR-Dependent Mechanism. *Neuron*, *5*, 801–808. https://doi.org/10.1016/j.neuron.2012.03.035

Huang, Y., Yoon, K., Ko, H., Jiao, S., Ito, W., Wu, J.-Y., Yung, W.-H., Lu, B., & Morozov, A. (2016). 5-HT3a Receptors Modulate Hippocampal Gamma Oscillations by Regulating Synchrony of Parvalbumin-Positive Interneurons. *Cerebral Cortex*, *26*(2), 576–585. https://doi.org/10.1093/cercor/bhu209

Humphreys, G. I., Ziegler, Y. S., & Nardulli, A. M. (2014). 17β-Estradiol Modulates Gene Expression in the Female Mouse Cerebral Cortex. *PLoS ONE*, *9*(11), e111975. https://doi.org/10.1371/journal.pone.0111975

Hussain, D., Hanafi, S., Konishi, K., Brake, W. G., & Bohbot, V. D. (2016). Modulation of spatial and response strategies by phase of the menstrual cycle in women tested in a virtual navigation task. *Psychoneuroendocrinology*, *70*, 108–117. https://doi.org/10.1016/j.psyneuen.2016.05.008

Hussain, D., Shams, W. M., & Brake, W. (2014). Estrogen and memory system bias in females across the lifespan. *Translational Neuroscience*, *5*(1), 35–50. https://doi.org/10.2478/s13380-014-0209-7

Hutter, J. A., & Chapman, C. A. (2013). Exposure to cues associated with palatable food reward results in a dopamine D2 receptor-dependent suppression of evoked synaptic responses in the entorhinal cortex. *Behavioral and Brain Functions*, *9*(1), 37. https://doi.org/10.1186/1744-9081-9-37

Hwang, W. J., Lee, T. Y., Kim, N. S., & Kwon, J. S. (2020). The Role of Estrogen Receptors and Their Signaling across Psychiatric Disorders. *International Journal of Molecular Sciences*, *22*(1), 373. https://doi.org/10.3390/ijms22010373

Ichikawa, T., Ajiki, K., Matsuura, J., & Misawa, H. (1997). Localization of two cholinergic markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: in situ hybridization histochemistry and immunohistochemistry. *Journal of Chemical Neuroanatomy*, *13*(1), 23–39. https://doi.org/10.1016/s0891-0618(97)00021-5

Jacome, L. F., Gautreaux, C., Inagaki, T., Mohan, G., Alves, S., Lubbers, L. S., & Luine, V. (2010). Estradiol and ERβ agonists enhance recognition memory, and DPN, an ERβ agonist, alters brain monoamines. *Neurobiology of Learning and Memory*, *94*(4), 488–498. https://doi.org/10.1016/j.nlm.2010.08.016

Jossin, Y. (2020). Reelin Functions, Mechanisms of Action and Signaling Pathways During Brain Development and Maturation. *Biomolecules*, *10*(6), 964. https://doi.org/10.3390/biom10060964

Kadish, I., & Groen, T. van. (2002). Low Levels of Estrogen Significantly Diminish Axonal Sprouting after Entorhinal Cortex Lesions in the Mouse. *Journal of Neuroscience*, *22*(10), 4095–4102. https://doi.org/10.1523/jneurosci.22-10-04095.2002

Kato, A., Hojo, Y., Higo, S., Komatsuzaki, Y., Murakami, G., Yoshino, H., Uebayashi, M., & Kawato, S. (2013). Female hippocampal estrogens have a significant correlation with cyclic fluctuation of hippocampal spines. *Frontiers in Neural Circuits*, *7*, 149. https://doi.org/10.3389/fncir.2013.00149

Kecskés, M., Henn-Mike, N., Agócs-Laboda, Á., Szőcs, S., Petykó, Z., & Varga, C. (2020). Somatostatin expressing GABAergic interneurons in the medial entorhinal cortex preferentially inhibit layerIII-V pyramidal cells. *Communications Biology*, *3*(1), 754. https://doi.org/10.1038/s42003-020-01496-x

Khan, M. M., Dhandapani, K. M., Zhang, Q., & Brann, D. W. (2013). Estrogen regulation of spine density and excitatory synapses in rat prefrontal and somatosensory cerebral cortex. *Steroids*, *78*(6), 614–623. https://doi.org/10.1016/j.steroids.2012.12.005

Khan, U. A., Liu, L., Provenzano, F. A., Berman, D. E., Profaci, C. P., Sloan, R., Mayeux, R., Duff, K. E., & Small, S. A. (2014). Molecular drivers and cortical spread of lateral entorhinal cortex dysfunction in preclinical Alzheimer's disease. *Nature Neuroscience*, *17*(2), 304–311. https://doi.org/10.1038/nn.3606

Kim, M. T., Soussou, W., Gholmieh, G., Ahuja, A., Tanguay, A., Berger, T. W., & Brinton, R. D. (2006). 17β-Estradiol potentiates field excitatory postsynaptic potentials within each subfield of the hippocampus with greatest potentiation of the associational/commissural afferents of CA3. *Neuroscience*, *141*(1), 391–406. https://doi.org/10.1016/j.neuroscience.2006.03.075

Kiss, Á., Delattre, A. M., Pereira, S. I. R., Carolino, R. G., Szawka, R. E., Anselmo-Franci, J. A., Zanata, S. M., & Ferraz, A. C. (2012). 17β-Estradiol replacement in young, adult and middle-aged female ovariectomized rats promotes improvement of spatial reference memory and an antidepressant effect and alters monoamines and BDNF levels in memory- and depression-related brain areas. *Behavioural Brain Research*, *227*(1), 100–108. https://doi.org/10.1016/j.bbr.2011.10.047

Klink, R., & Alonso, A. (1997). Muscarinic Modulation of the Oscillatory and Repetitive Firing Properties of Entorhinal Cortex Layer II Neurons. *Journal of Neurophysiology*, *77*(4), 1813–1828. https://doi.org/10.1152/jn.1997.77.4.1813

Knierim, J. J., Neunuebel, J. P., & Deshmukh, S. S. (2013). Functional correlates of the lateral and medial entorhinal cortex: objects, path integration and local-global reference frames. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1635), 20130369–20130369. https://doi.org/10.1098/rstb.2013.0369

Koch, T., Jørgensen, J. T., Christensen, J., Duun-Henriksen, A. K., Priskorn, L., Simonsen, M. K., Dehlendorff, C., Andersen, Z. J., Juul, A., Bräuner, E. V., & Hickey, M. (2022). Bilateral oophorectomy and rate of colorectal cancer: A prospective cohort study. *International Journal of Cancer*, *150*(1), 38–46. https://doi.org/10.1002/ijc.33776

Koebele, S. V., & Bimonte-Nelson, H. A. (2016). Modeling menopause: The utility of rodents in translational behavioral endocrinology research. *Maturitas*, *87*, 5–17. https://doi.org/10.1016/j.maturitas.2016.01.015

Koebele, S. V., Mennenga, S. E., Hiroi, R., Quihuis, A. M., Hewitt, L. T., Poisson, M. L., George, C., Mayer, L. P., Dyer, C. A., Aiken, L. S., Demers, L. M., Carson, C., & Bimonte-Nelson, H. A. (2017). Cognitive changes across the menopause transition: A longitudinal evaluation of the impact of age and ovarian status on spatial memory. *Hormones and Behavior*, *87*, 96–114. https://doi.org/10.1016/j.yhbeh.2016.10.010

Kompoliti, K., Chu, Y., Polish, A., Roberts, J., McKay, H., Mufson, E. J., Leurgans, S., Morrison, J. H., & Kordower, J. H. (2004). Effects of estrogen replacement therapy on cholinergic basal forebrain neurons and cortical cholinergic innervation in young and aged

ovariectomized rhesus monkeys. *Journal of Comparative Neurology*, *472*(2), 193–207. https://doi.org/10.1002/cne.20050

Kondo, H., & Zaborszky, L. (2016). Topographic organization of the basal forebrain projections to the perirhinal, postrhinal, and entorhinal cortex in rats. *Journal of Comparative Neurology*, *524*(12), 2503–2515. https://doi.org/10.1002/cne.23967

Korol, D. L., & Kolo, L. L. (2002). Estrogen-induced changes in place and response learning in young adult female rats. *Behavioral Neuroscience*, *116*(3), 411. https://doi.org/10.1037/0735-7044.116.3.411

Korol, D. L., Malin, E. L., Borden, K. A., Busby, R. A., & Couper-Leo, J. (2004). Shifts in preferred learning strategy across the estrous cycle in female rats. *Hormones and Behavior*, *45*(5), 330–338. https://doi.org/10.1016/j.yhbeh.2004.01.005

Kritzer, M. F. (2002). Regional, Laminar, and Cellular Distribution of Immunoreactivity for ERα and ERβ in the Cerebral Cortex of Hormonally Intact, Adult Male and Female Rats. *Cerebral Cortex*, *12*(2), 116–128. https://doi.org/10.1093/cercor/12.2.116

Kumar, A., & Foster, T. C. (2020). G Protein-Coupled Estrogen Receptor: Rapid Effects on Hippocampal-Dependent Spatial Memory and Synaptic Plasticity. *Frontiers in Endocrinology*, *11*, 385. https://doi.org/10.3389/fendo.2020.00385

Kumar, A., Bean, L. A., Rani, A., Jackson, T., & Foster, T. C. (2015). Contribution of estrogen receptor subtypes, ERα, ERβ, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. *Hippocampus*, *25*(12), 1556–1566. https://doi.org/10.1002/hipo.22475

Kuruvilla, M. V., & Ainge, J. A. (2017). Lateral Entorhinal Cortex Lesions Impair Local Spatial Frameworks. *Frontiers in Systems Neuroscience*, *11*, 30. https://doi.org/10.3389/fnsys.2017.00030

Leitner, F. C., Melzer, S., Lütcke, H., Pinna, R., Seeburg, P. H., Helmchen, F., & Monyer, H. (2016). Spatially segregated feedforward and feedback neurons support differential odor processing in the lateral entorhinal cortex. *Nature Neuroscience*, *19*(7), 935–944. https://doi.org/10.1038/nn.4303

Leung, C., Cao, F., Nguyen, R., Joshi, K., Aqrabawi, A. J., Xia, S., Cortez, M. A., Snead, O. C., Kim, J. C., & Jia, Z. (2018). Activation of Entorhinal Cortical Projections to the Dentate Gyrus Underlies Social Memory Retrieval. *Cell Reports*, *23*(8), 2379–2391. https://doi.org/10.1016/j.celrep.2018.04.073

Levin, E. R. (2009). Plasma membrane estrogen receptors. *Trends in Endocrinology & Metabolism*, *20*(10), 477–482. https://doi.org/10.1016/j.tem.2009.06.009

Lim, L., Mi, D., Llorca, A., & Marín, O. (2018). Development and Functional Diversification of Cortical Interneurons. *Neuron*, *100*(2), 294–313. https://doi.org/10.1016/j.neuron.2018.10.009

Lipton, P., & Eichenbaum, H. (2008). Complementary Roles of Hippocampus and Medial Entorhinal Cortex in Episodic Memory. *Neural Plasticity*, *2008*, 258467. https://doi.org/10.1155/2008/258467

Long, T., Yao, J. K., Li, J., Kirshner, Z. Z., Nelson, D., Dougherty, G. G., & Gibbs, R. B. (2019). Estradiol and selective estrogen receptor agonists differentially affect brain monoamines and amino acids levels in transitional and surgical menopausal rat models. *Molecular and Cellular Endocrinology*, *496*, 110533. https://doi.org/10.1016/j.mce.2019.110533

Lopez-Rojas, J., Solis, C. A. de, Leroy, F., Kandel, E. R., & Siegelbaum, S. A. (2022). A direct lateral entorhinal cortex to hippocampal CA2 circuit conveys social information required for social memory. *Neuron*. https://doi.org/10.1016/j.neuron.2022.01.028

Lu, C. -L., & Herndon, C. (2017). New roles for neuronal estrogen receptors. *Neurogastroenterology & Motility*, *29*(7), e13121. https://doi.org/10.1111/nmo.13121

Luine, V. N. (1985). Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. *Experimental Neurology*, *89*(2), 484–490. https://doi.org/10.1016/0014-4886(85)90108-6

MacLennan, A. H., Henderson, V. W., Paine, B. J., Mathias, J., Ramsay, E. N., Ryan, P., Stocks, N. P., & Taylor, A. W. (2006). Hormone therapy, timing of initiation, and cognition in women aged older than 60 years: the REMEMBER pilot study. *Menopause*, *13*(1), 28–36. https://doi.org/10.1097/01.gme.0000191204.38664.61

Maioli, S., Leander, K., Nilsson, P., & Nalvarte, I. (2021). Estrogen receptors and the aging brain. *Essays in Biochemistry*, *65*(6), 913–925. https://doi.org/10.1042/ebc20200162

Maki, P. M. (2006). Hormone therapy and cognitive function: Is there a critical period for benefit? *Neuroscience*, *138*(3), 1027–1030. https://doi.org/10.1016/j.neuroscience.2006.01.001

Maki, P. M., Rich, J. B., & Rosenbaum, R. S. (2002). Implicit memory varies across the menstrual cycle: estrogen effects in young women. *Neuropsychologia*, *40*(5), 518–529. https://doi.org/10.1016/s0028-3932(01)00126-9

Mannino, C. A., South, S. M., Inturrisi, C. E., & Quinones-Jenab, V. (2005). Pharmacokinetics and Effects of 17β-Estradiol and Progesterone Implants in Ovariectomized Rats. *The Journal of Pain*, *6*(12), 809–816. https://doi.org/10.1016/j.jpain.2005.07.007

Mans, R. A., Warmus, B. A., Smith, C. C., & McMahon, L. L. (2014). An acetylcholinesterase inhibitor, eserine, induces long-term depression at CA3-CA1 synapses in the hippocampus of

adult rats. *Journal of Neurophysiology*, *112*(10), 2388–2397. https://doi.org/10.1152/jn.00048.2014

Marraudino, M., Carrillo, B., Bonaldo, B., Llorente, R., Campioli, E., Garate, I., Pinos, H., Garcia-Segura, L. M., Collado, P., & Grassi, D. (2021). G Protein-Coupled Estrogen Receptor Immunoreactivity in the Rat Hypothalamus Is Widely Distributed in Neurons, Astrocytes, and Oligodendrocytes, Fluctuates during the Estrous Cycle, and Is Sexually Dimorphic. *Neuroendocrinology*, *111*(7), 660–677. https://doi.org/10.1159/000509583

Matsuda, K., Sakamoto, H., Mori, H., Hosokawa, K., Kawamura, A., Itose, M., Nishi, M., Prossnitz, E. R., & Kawata, M. (2008). Expression and intracellular distribution of the G protein-coupled receptor 30 in rat hippocampal formation. *Neuroscience Letters*, *441*(1), 94–99. https://doi.org/10.1016/j.neulet.2008.05.108

Matyi, J. M., Rattinger, G. B., Schwartz, S., Buhusi, M., & Tschanz, J. T. (2019). Lifetime estrogen exposure and cognition in late life: the Cache County Study. *Menopause*, *26*(12), 1366–1374. https://doi.org/10.1097/gme.0000000000001405

McMillan, P., Singer, C., & Dorsa, D. (1996). The effects of ovariectomy and estrogen replacement on trkA and choline acetyltransferase mRNA expression in the basal forebrain of the adult female Sprague-Dawley rat. *Journal of Neuroscience*, *16*(5), 1860–1865. https://doi.org/10.1523/jneurosci.16-05-01860.1996

McNeil, M. A., & Merriam, S. B. (2021). Menopause. *Annals of Internal Medicine*, *174*(7), ITC97–ITC112. https://doi.org/10.7326/aitc202107200

Merchenthaler, I., Lane, M. V., Numan, S., & Dellovade, T. L. (2004). Distribution of estrogen receptor α and β in the mouse central nervous system: In vivo autoradiographic and immunocytochemical analyses. *Journal of Comparative Neurology*, *473*(2), 270–291. https://doi.org/10.1002/cne.20128

Micevych, P., & Sinchak, K. (2008). Estradiol regulation of progesterone synthesis in the brain. *Molecular and Cellular Endocrinology*, *290*(1–2), 44–50. https://doi.org/10.1016/j.mce.2008.04.016

Milad, M. R., Igoe, S. A., Lebron-Milad, K., & Novales, J. E. (2009). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience*, *164*(3), 887–895. https://doi.org/10.1016/j.neuroscience.2009.09.011

Mordecai, K. L., Rubin, L. H., & Maki, P. M. (2008). Effects of menstrual cycle phase and oral contraceptive use on verbal memory. *Hormones and Behavior*, *54*(2), 286–293. https://doi.org/10.1016/j.yhbeh.2008.03.006

Morel, G. R., Andersen, T., Pardo, J., Zuccolilli, G. O., Cambiaggi, V. L., Hereñú, C. B., & Goya, R. G. (2015). Cognitive impairment and morphological changes in the dorsal

hippocampus of very old female rats. *Neuroscience*, *303*, 189–199. https://doi.org/10.1016/j.neuroscience.2015.06.050

Moser, V. A., Workman, M. J., Hurwitz, S. J., Lipman, R. M., Pike, C. J., & Svendsen, C. N. (2021). Microglial transcription profiles in mouse and human are driven by APOE4 and sex. *IScience*, *24*(11), 103238. https://doi.org/10.1016/j.isci.2021.103238

Mukherjee, J., Cardarelli, R. A., Cantaut-Belarif, Y., Deeb, T. Z., Srivastava, D. P., Tyagarajan, S. K., Pangalos, M. N., Triller, A., Maguire, J., Brandon, N. J., & Moss, S. J. (2017). Estradiol modulates the efficacy of synaptic inhibition by decreasing the dwell time of GABAA receptors at inhibitory synapses. *Proceedings of the National Academy of Sciences*, *114*(44), 11763–11768. https://doi.org/10.1073/pnas.1705075114

Murphy, D. D., Cole, N. B., Greenberger, V., & Segal, M. (1998). Estradiol Increases Dendritic Spine Density by Reducing GABA Neurotransmission in Hippocampal Neurons. *Journal of Neuroscience*, *18*(7), 2550–2559. https://doi.org/10.1523/jneurosci.18-07-02550.1998

Newhouse, P., & Dumas, J. (2015). Estrogen–cholinergic interactions: Implications for cognitive aging. *Hormones and Behavior*, *74*, 173–185. https://doi.org/10.1016/j.yhbeh.2015.06.022

Nielsen, S. E., Ahmed, I., & Cahill, L. (2013). Sex and menstrual cycle phase at encoding influence emotional memory for gist and detail. *Neurobiology of Learning and Memory*, *106*, 56–65. https://doi.org/10.1016/j.nlm.2013.07.015

Nilssen, E. S., Doan, T. P., Nigro, M. J., Ohara, S., & Witter, M. P. (2019). Neurons and networks in the entorhinal cortex: A reappraisal of the lateral and medial entorhinal subdivisions mediating parallel cortical pathways. *Hippocampus*, *29*(12), 1238–1254. https://doi.org/10.1002/hipo.23145

Nilssen, E. S., Jacobsen, B., Fjeld, G., Nair, R. R., Blankvoort, S., Kentros, C., & Witter, M. P. (2018). Inhibitory Connectivity Dominates the Fan Cell Network in Layer II of Lateral Entorhinal Cortex. *Journal of Neuroscience*, *38*(45), 9712–9727. https://doi.org/10.1523/jneurosci.1290-18.2018

Nin, M. S., Martinez, L. A., Pibiri, F., Nelson, M., & Pinna, G. (2011). Neurosteroids Reduce Social Isolation-Induced Behavioral Deficits: A Proposed Link with Neurosteroid-Mediated Upregulation of BDNF Expression. *Frontiers in Endocrinology*, *2*, 73. https://doi.org/10.3389/fendo.2011.00073

Norbury, R., Travis, M. J., Erlandsson, K., Waddington, W., Ell, P. J., & Murphy, D. G. M. (2007). Estrogen Therapy and brain muscarinic receptor density in healthy females: A SPET study. *Hormones and Behavior*, *51*(2), 249–257. https://doi.org/10.1016/j.yhbeh.2006.10.007

Nuñez, A., & Buño, W. (2021). The Theta Rhythm of the Hippocampus: From Neuronal and Circuit Mechanisms to Behavior. *Frontiers in Cellular Neuroscience*, *15*, 649262. https://doi.org/10.3389/fncel.2021.649262

Oberlander, J. G., & Woolley, C. S. (2016). 17β-Estradiol Acutely Potentiates Glutamatergic Synaptic Transmission in the Hippocampus through Distinct Mechanisms in Males and Females. *The Journal of Neuroscience*, *36*(9), 2677–2690. https://doi.org/10.1523/jneurosci.4437-15.2016

Ohara, S., Onodera, M., Simonsen, Ø. W., Yoshino, R., Hioki, H., Iijima, T., Tsutsui, K.-I., & Witter, M. P. (2018). Intrinsic Projections of Layer Vb Neurons to Layers Va, III, and II in the Lateral and Medial Entorhinal Cortex of the Rat. *Cell Reports*, *24*(1), 107–116. https://doi.org/10.1016/j.celrep.2018.06.014

Olajide, O. J., & Chapman, C. A. (2021). Amyloid-β (1-42) peptide induces rapid NMDA receptor-dependent alterations at glutamatergic synapses in the entorhinal cortex. *Neurobiology of Aging*, *105*, 296–309. https://doi.org/10.1016/j.neurobiolaging.2021.05.006

Orozco, L. J., Tristan, M., Vreugdenhil, M. M., & Salazar, A. (2014). Hysterectomy versus hysterectomy plus oophorectomy for premenopausal women. *Cochrane Database of Systematic Reviews*, *7*, CD005638. https://doi.org/10.1002/14651858.cd005638.pub3

Pala, S., Atilgan, R., Kuloglu, T., Yalçın, E., Kaya, N., & Etem, E. (2019). The decrease in hippocampal transient receptor potential M2 (TRPM2) channel and muscarinic acetylcholine receptor 1 (CHRM1) is associated with memory loss in a surgical menopause rat model. *Archives of Medical Science : AMS*, *17*(1), 228–235. https://doi.org/10.5114/aoms.2019.83760

Parsons, B., Maclusky, N. J., Krey, L., Pfaff, D. W., & McEwen, B. S. (1980). The Temporal Relationship between Estrogen-Inducible Progestin Receptors in the Female Rat Brain and the Time Course of Estrogen Activation of Mating Behavior*. *Endocrinology*, *107*(3), 774–779. https://doi.org/10.1210/endo-107-3-774

Patel, S. A., Frick, K. M., Newhouse, P. A., & Astur, R. S. (2022). Estradiol effects on spatial memory in women. *Behavioural Brain Research*, *417*, 113592. https://doi.org/10.1016/j.bbr.2021.113592

Paxinos, G., & Watson, R. (1998). *The rat brain in stereotaxic coordinates* (3rd ed.). Academic Press.

Peragine, D. E., Gervais, N. J., Simeon-Spezzaferro, C., & Einstein, G. (2022). A new angle on mental rotation ability in transgender men: Modulation by ovarian milieu. *Psychoneuroendocrinology*, *141*, 105751. https://doi.org/10.1016/j.psyneuen.2022.105751

Peragine, D., Simeon-Spezzaferro, C., Brown, A., Gervais, N. J., Hampson, E., & Einstein, G. (2020). Sex difference or hormonal difference in mental rotation? The influence of ovarian milieu. *Psychoneuroendocrinology*, *115*, 104488. https://doi.org/10.1016/j.psyneuen.2019.104488

Phillips, S. M., & Sherwin, B. B. (1992). Effects of estrogen on memory function in surgically menopausal women. *Psychoneuroendocrinology*, *17*(5), 485–495. https://doi.org/10.1016/0306-4530(92)90007-t

Ping, S. E., Trieu, J., Wlodek, M. E., & Barrett, G. L. (2008). Effects of estrogen on basal forebrain cholinergic neurons and spatial learning. *Journal of Neuroscience Research*, *86*(7), 1588–1598. https://doi.org/10.1002/jnr.21609

Pisansky, M. T., Lefevre, E. M., Retzlaff, C. L., Trieu, B. H., Leipold, D. W., & Rothwell, P. E. (2019). Nucleus Accumbens Fast-Spiking Interneurons Constrain Impulsive Action. *Biological Psychiatry*, *86*(11), 836–847. https://doi.org/10.1016/j.biopsych.2019.07.002

Pompili, A., Tomaz, C., Arnone, B., Tavares, M. C., & Gasbarri, A. (2010). Working and reference memory across the estrous cycle of rat: A long-term study in gonadally intact females. *Behavioural Brain Research*, *213*(1), 10–18. https://doi.org/10.1016/j.bbr.2010.04.018

Pozzo-Miller, L. D., Inoue, T., & Murphy, D. D. (1999). Estradiol Increases Spine Density and NMDA-Dependent Ca2+ Transients in Spines of CA1 Pyramidal Neurons From Hippocampal Slices. *Journal of Neurophysiology*, *81*(3), 1404–1411. https://doi.org/10.1152/jn.1999.81.3.1404

Quinlan, M. G., Hussain, D., & Brake, W. G. (2008). Use of cognitive strategies in rats: The role of estradiol and its interaction with dopamine. *Hormones and Behavior*, *53*(1), 185–191. https://doi.org/10.1016/j.yhbeh.2007.09.015

Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., & Prossnitz, E. R. (2005). A Transmembrane Intracellular Estrogen Receptor Mediates Rapid Cell Signaling. *Science*, *307*(5715), 1625–1630. https://doi.org/10.1126/science.1106943

Rocca, W. A., Grossardt, B. R., & Shuster, L. T. (2014). Oophorectomy, estrogen, and dementia: A 2014 update. *Molecular and Cellular Endocrinology*, *389*(1–2), 7–12. https://doi.org/10.1016/j.mce.2014.01.020

Rodo, C., Sargolini, F., & Save, E. (2017). Processing of spatial and non-spatial information in rats with lesions of the medial and lateral entorhinal cortex: Environmental complexity matters. *Behavioural Brain Research*, *320*, 200–209. https://doi.org/10.1016/j.bbr.2016.12.009

Rose'Meyer, R. B., Mellick, A. S., Garnham, B. G., Harrison, G. J., Massa, H. M., & Griffiths, L. R. (2003). The measurement of adenosine and estrogen receptor expression in rat brains following ovariectomy using quantitative PCR analysis. *Brain Research Protocols*, *11*(1), 9–18. https://doi.org/10.1016/s1385-299x(02)00219-2

Rudick, C. N., Gibbs, R. B., & Woolley, C. S. (2003). A Role for the Basal Forebrain Cholinergic System in Estrogen-Induced Disinhibition of Hippocampal Pyramidal Cells.

Journal of Neuroscience, *23*(11), 4479–4490. https://doi.org/10.1523/jneurosci.23-11-04479.2003

Sanders, H., Rennó-Costa, C., Idiart, M., & Lisman, J. (2015). Grid Cells and Place Cells: An Integrated View of their Navigational and Memory Function. *Trends in Neurosciences*, *38*(12), 763–775. https://doi.org/10.1016/j.tins.2015.10.004

Santoro, N., Roeca, C., Peters, B. A., & Neal-Perry, G. (2020). The Menopause Transition: Signs, Symptoms, and Management Options. *The Journal of Clinical Endocrinology & Metabolism*, *106*(1), 1–15. https://doi.org/10.1210/clinem/dgaa764

Sárvári, M., Hrabovszky, E., Kalló, I., Solymosi, N., Tóth, K., Likó, I., Széles, J., Mahó, S., Molnár, B., & Liposits, Z. (2011). Estrogens regulate neuroinflammatory genes via estrogen receptors α and β in the frontal cortex of middle-aged female rats. *Journal of Neuroinflammation*, *8*(1), 82. https://doi.org/10.1186/1742-2094-8-82

Schindler, A. E. (2015). *Progestogens in Obstetrics and Gynecology*. 33–40. https://doi.org/10.1007/978-3-319-14385-9_2

Schumacher, M., Mattern, C., Ghoumari, A., Oudinet, J. P., Liere, P., Labombarda, F., Sitruk-Ware, R., Nicola, A. F. D., & Guennoun, R. (2014). Revisiting the roles of progesterone and allopregnanolone in the nervous system: Resurgence of the progesterone receptors. *Progress in Neurobiology*, *113*, 6–39. https://doi.org/10.1016/j.pneurobio.2013.09.004

Schwarz, D. S., & Blower, M. D. (2016). The endoplasmic reticulum: structure, function and response to cellular signaling. *Cellular and Molecular Life Sciences*, *73*(1), 79–94. https://doi.org/10.1007/s00018-015-2052-6

Shalinsky, M. H., Magistretti, J., Ma, L., & Alonso, A. A. (2002). Muscarinic Activation of a Cation Current and Associated Current Noise in Entorhinal-Cortex Layer-II Neurons. *Journal of Neurophysiology*, *88*(3), 1197–1211. https://doi.org/10.1152/jn.2002.88.3.1197

Sherwin, B. B. (2009). Estrogen therapy: is time of initiation critical for neuroprotection? *Nature Reviews Endocrinology*, *5*(11), nrendo.2009.193. https://doi.org/10.1038/nrendo.2009.193

Shima, N., Yamaguchi, Y., & Yuri, K. (2002). Distribution of estrogen receptor β mRNA-containing cells in ovariectomized and estrogen-treated female rat brain. *Anatomical Science International*, *78*(2), 85. https://doi.org/10.1046/j.0022-7722.2003.00042.x

Shughrue, P. J., & Merchenthaler, I. (2001). Distribution of estrogen receptor β immunoreactivity in the rat central nervous system. *Journal of Comparative Neurology*, *436*(1), 64–81. https://doi.org/10.1002/cne.1054

Shughrue, P. J., Lane, M. V., & Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. *Journal of Comparative*

Neurology, *388*(4), 507–525. https://doi.org/10.1002/(sici)1096-9861(19971201)388:4<507::aid-cne1>3.0.co;2-6

Singh, M., Meyer, E. M., Millard, W. J., & Simpkins, J. W. (1994). Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. *Brain Research*, *644*(2), 305–312. https://doi.org/10.1016/0006-8993(94)91694-2

Smejkalova, T., & Woolley, C. S. (2010). Estradiol Acutely Potentiates Hippocampal Excitatory Synaptic Transmission through a Presynaptic Mechanism. *The Journal of Neuroscience*, *30*(48), 16137–16148. https://doi.org/10.1523/jneurosci.4161-10.2010

Solari, N., & Hangya, B. (2018). Cholinergic modulation of spatial learning, memory and navigation. *European Journal of Neuroscience*, *48*(5), 2199–2230. https://doi.org/10.1111/ejn.14089

Sommer, T., Richter, K., Singer, F., Derntl, B., Rune, G. M., Diekhof, E., & Bayer, J. (2018). Effects of the experimental administration of oral estrogen on prefrontal functions in healthy young women. *Psychopharmacology*, *235*(12), 3465–3477. https://doi.org/10.1007/s00213-018-5061-y

Song, Y.-H., Yoon, J., & Lee, S.-H. (2021). The role of neuropeptide somatostatin in the brain and its application in treating neurological disorders. *Experimental & Molecular Medicine*, *53*(3), 328–338. https://doi.org/10.1038/s12276-021-00580-4

Spencer, J. L., Waters, E. M., Romeo, R. D., Wood, G. E., Milner, T. A., & McEwen, B. S. (2008). Uncovering the mechanisms of estrogen effects on hippocampal function. *Frontiers in Neuroendocrinology*, *29*(2), 219–237. https://doi.org/10.1016/j.yfrne.2007.08.006

Stelly, C. E., Cronin, J., Daniel, J. M., & Schrader, L. A. (2012). Long-Term Oestradiol Treatment Enhances Hippocampal Synaptic Plasticity that is Dependent on Muscarinic Acetylcholine Receptors in Ovariectomised Female Rats. *Journal of Neuroendocrinology*, *24*(6), 887–896. https://doi.org/10.1111/j.1365-2826.2012.02287.x

Tabatadze, N., Huang, G., May, R. M., Jain, A., & Woolley, C. S. (2015). Sex Differences in Molecular Signaling at Inhibitory Synapses in the Hippocampus. *The Journal of Neuroscience*, *35*(32), 11252–11265. https://doi.org/10.1523/JNEUROSCI.1067-15.2015

Tahvildari, B., & Alonso, A. (2005). Morphological and electrophysiological properties of lateral entorhinal cortex layers II and III principal neurons. *Journal of Comparative Neurology*, *491*(2), 123–140. https://doi.org/10.1002/cne.20706

Tanaka, M., & Sokabe, M. (2013). Bidirectional modulatory effect of 17β-estradiol on NMDA receptors via ERα and ERβ in the dentate gyrus of juvenile male rats. *Neuropharmacology*, *75*, 262–273. https://doi.org/10.1016/j.neuropharm.2013.07.029

Taxier, L. R., Gross, K. S., & Frick, K. M. (2020). Oestradiol as a neuromodulator of learning and memory. *Nature Reviews Neuroscience*, *21*(10), 535–550. https://doi.org/10.1038/s41583-020-0362-7

Tian, Z., Wang, Y., Zhang, N., Guo, Y., Feng, B., Liu, S., & Zhao, M. (2013). Estrogen receptor GPR30 exerts anxiolytic effects by maintaining the balance between GABAergic and glutamatergic transmission in the basolateral amygdala of ovariectomized mice after stress. *Psychoneuroendocrinology*, *38*(10), 2218–2233. https://doi.org/10.1016/j.psyneuen.2013.04.011

Tinkler, G., Tobin, J., & Voytko, M. (2004). Effects of two years of estrogen loss or replacement on nucleus basalis cholinergic neurons and cholinergic fibers to the dorsolateral prefrontal and inferior parietal cortex of monkeys. *Journal of Comparative Neurology*, *469*(4), 507–521. https://doi.org/10.1002/cne.11028

Triggle, D. J., Mitchell, J. M., & Filler, R. (1998). The Pharmacology of Physostigmine. *CNS Drug Reviews*, *4*(2), 87–136. https://doi.org/10.1111/j.1527-3458.1998.tb00059.x

Uddin, Md. S., Rahman, Md. M., Jakaria, Md., Rahman, Md. S., Hossain, Md. S., Islam, A., Ahmed, M., Mathew, B., Omar, U. M., Barreto, G. E., & Ashraf, G. M. (2020). Estrogen Signaling in Alzheimer's Disease: Molecular Insights and Therapeutic Targets for Alzheimer's Dementia. *Molecular Neurobiology*, *57*(6), 2654–2670. https://doi.org/10.1007/s12035-020-01911-8

Uzum, G., Bahcekapili, N., Baltaci, A. K., Mogulkoc, R., & Ziylan, Y. Z. (2016). Chronic (3-Weeks) Treatment of Estrogen (17β-Estradiol) Enhances Working and Reference Memory in Ovariectomized Rats: Role of Acetylcholine. *Neurochemical Research*, *41*(6), 1468–1474. https://doi.org/10.1007/s11064-016-1858-4

Vajaria, R., & Vasudevan, N. (2018). Is the membrane estrogen receptor, GPER1, a promiscuous receptor that modulates nuclear estrogen receptor-mediated functions in the brain? *Hormones and Behavior*, *104*, 165–172. https://doi.org/10.1016/j.yhbeh.2018.06.012

Van Cauter, T., Camon, J., Alvernhe, A., Elduayen, C., Sargolini, F., & Save, E. (2013). Distinct Roles of Medial and Lateral Entorhinal Cortex in Spatial Cognition. *Cerebral Cortex*, *23*(2), 451–459. https://doi.org/10.1093/cercor/bhs033

Vaucher, E., Reymond, I., Najaffe, R., Kar, S., Quirion, R., Miller, M. M., & Franklin, K. B. J. (2002). Estrogen effects on object memory and cholinergic receptors in young and old female mice. *Neurobiology of Aging*, *23*(1), 87–95. https://doi.org/10.1016/s0197-4580(01)00250-0

Velíšková, J., & Velíšek, L. (2013). Gonadal status-dependent effects of in vivo β-estradiol administration to female rats on in vitro epileptiform activity induced by low [Mg2+]o in combined hippocampus-entorhinal cortex slices. *Epilepsy Research*, *107*(3), 297–301. https://doi.org/10.1016/j.eplepsyres.2013.09.002

Verma, A., & Sharma, S. (2015). Beneficial Effect of Protein Tyrosine Phosphatase Inhibitor and Phytoestrogen in Dyslipidemia-Induced Vascular Dementia in Ovariectomized Rats. *Journal of Stroke and Cerebrovascular Diseases*, *24*(11), 2434–2446. https://doi.org/10.1016/j.jstrokecerebrovasdis.2015.02.019

Vrtačnik, P., Ostanek, B., Mencej-Bedrač, S., & Marc, J. (2014). The many faces of estrogen signaling. *Biochemia Medica*, *24*(3), 329–342. https://doi.org/10.11613/bm.2014.035

Wang, J., Yu, R., Han, Q.-Q., Huang, H.-J., Wang, Y.-L., Li, H.-Y., Wang, H.-M., Chen, X.-R., Ma, S.-L., & Yu, J. (2018). G-1 exhibit antidepressant effect, increase of hippocampal ERs expression and improve hippocampal redox status in aged female rats. *Behavioural Brain Research*, *Arch Gen Psychiatry 63 4 2006*. https://doi.org/10.1016/j.bbr.2018.07.017

Wang, M. (2011). Neurosteroids and GABA-A Receptor Function. *Frontiers in Endocrinology*, *2*, 44. https://doi.org/10.3389/fendo.2011.00044

Waters, E. M., Thompson, L. I., Patel, P., Gonzales, A. D., Ye, H. (Zhiyu), Filardo, E. J., Clegg, D. J., Gorecka, J., Akama, K. T., McEwen, B. S., & Milner, T. A. (2015). G-Protein-Coupled Estrogen Receptor 1 Is Anatomically Positioned to Modulate Synaptic Plasticity in the Mouse Hippocampus. *The Journal of Neuroscience*, *35*(6), 2384–2397. https://doi.org/10.1523/jneurosci.1298-14.2015

Weiser, M. J., Wu, T. J., & Handa, R. J. (2008). Estrogen Receptor-β Agonist Diarylpropionitrile: Biological Activities of R- and S-Enantiomers on Behavior and Hormonal Response to Stress. *Endocrinology*, *150*(4), 1817–1825. https://doi.org/10.1210/en.2008-1355

Widholm, O., Kantero, R. -L., Axelson, E., Johansson, E. D. B., & Wide, L. (1974). Endocrine Changes Before and After the Menarche. *Acta Obstetricia et Gynecologica Scandinavica*, *53*(3), 197–208. https://doi.org/10.3109/00016347409162157

Witter, M. P., Doan, T. P., Jacobsen, B., Nilssen, E. S., & Ohara, S. (2017). Architecture of the Entorhinal Cortex A Review of Entorhinal Anatomy in Rodents with Some Comparative Notes. *Frontiers in Systems Neuroscience*, *11*, 46. https://doi.org/10.3389/fnsys.2017.00046

Wong, M., & Moss, R. (1992). Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. *Journal of Neuroscience*, *12*(8), 3217–3225. https://doi.org/10.1523/jneurosci.12-08-03217.1992

Woolf, N. J., & Butcher, L. L. (2011). Cholinergic systems mediate action from movement to higher consciousness. *Behavioural Brain Research*, *221*(2), 488–498. https://doi.org/10.1016/j.bbr.2009.12.046

Woolley, C. S., & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *Journal of Neuroscience*, *12*(7), 2549–2554. https://doi.org/10.1523/jneurosci.12-07-02549.1992

Woolley, C. S., & McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *Journal of Comparative Neurology*, *336*(2), 293–306. https://doi.org/10.1002/cne.903360210

Xu, Y., Sheng, H., Tang, Z., Lu, J., & Ni, X. (2015). Inflammation and increased IDO in hippocampus contribute to depression-like behavior induced by estrogen deficiency. *Behavioural Brain Research*, *288*, 71–78. https://doi.org/10.1016/j.bbr.2015.04.017

Yagi, S., Drewczynski, D., Wainwright, S. R., Barha, C. K., Hershorn, O., & Galea, L. A. M. (2017). Sex and estrous cycle differences in immediate early gene activation in the hippocampus and the dorsal striatum after the cue competition task. *Hormones and Behavior*, *87*, 69–79. https://doi.org/10.1016/j.yhbeh.2016.10.019

Yáñez, M., Gil-Longo, J., & Campos-Toimil, M. (2012). Calcium Signaling. *Advances in Experimental Medicine and Biology*, *740*, 461–482. https://doi.org/10.1007/978-94-007-2888-2_19

Yao, F., Zhuang, Y., Shen, X., & Wang, X. (2022). Attentional bias towards appealing and disgusting food cues varies with the menstrual cycle. *Appetite*, *175*, 106063. https://doi.org/10.1016/j.appet.2022.106063

Ye, J., Witter, M. P., Moser, M.-B., & Moser, E. I. (2018). Entorhinal fast-spiking speed cells project to the hippocampus. *Proceedings of the National Academy of Sciences*, *115*(7), 201720855. https://doi.org/10.1073/pnas.1720855115

Zhang, K., Pollack, S., Ghods, A., Dicken, C., Isaac, B., Adel, G., Zeitlian, G., & Santoro, N. (2008). Onset of Ovulation after Menarche in Girls: A Longitudinal Study. *The Journal of Clinical Endocrinology & Metabolism*, *93*(4), 1186–1194. https://doi.org/10.1210/jc.2007-1846

Zhang, Y., Xiao, X., Zhang, X.-M., Zhao, Z.-Q., & Zhang, Y.-Q. (2012). Estrogen Facilitates Spinal Cord Synaptic Transmission via Membrane-bound Estrogen Receptors IMPLICATIONS FOR PAIN HYPERSENSITIVITY*. *Journal of Biological Chemistry*, *287*(40), 33268–33281. https://doi.org/10.1074/jbc.m112.368142

Zhao, T., Ding, Q., Hu, J., He, S., Shi, F., & Ma, L. (2016). GPER expressed on microglia mediates the anti-inflammatory effect of estradiol in ischemic stroke. *Brain and Behavior*, *4*. https://doi.org/10.1002/brb3.449

Zhao, W., Hou, Y., Song, X., Wang, L., Zhang, F., Zhang, H., Yu, H., & Zhou, Y. (2021). Estrogen Deficiency Induces Mitochondrial Damage Prior to Emergence of Cognitive Deficits in a Postmenopausal Mouse Model. *Frontiers in Aging Neuroscience*, *13*, 713819. https://doi.org/10.3389/fnagi.2021.713819

Zhou, H., Yu, Z., Luo, L., Xie, F., Wang, Y., & Wan, Z. (2021). The effect of hormone replacement therapy on cognitive function in healthy postmenopausal women: a meta-

analysis of 23 randomized controlled trials. *Psychogeriatrics*, *21*(6), 926–938. https://doi.org/10.1111/psyg.12768

Zhvania, M. G., Japaridze, N., Tizabi, Y., Lomidze, N., Pochkhidze, N., & Lordkipanidze, T. (2021). Age-related cognitive decline in rats is sex and context dependent. *Neuroscience Letters*, *765*, 136262. https://doi.org/10.1016/j.neulet.2021.136262

Zoladz, P. R., Peters, D. M., Cadle, C. E., Kalchik, A. E., Aufdenkampe, R. L., Dailey, A. M., Brown, C. M., Scharf, A. R., Earley, M. B., Knippen, C. L., & Rorabaugh, B. R. (2015). Post-learning stress enhances long-term memory and differentially influences memory in females depending on menstrual stage. *Acta Psychologica*, *160*, 127–133. https://doi.org/10.1016/j.actpsy.2015.07.008