Estrogens Rapidly Enhance Neural Plasticity and Learning

by

Anna Phan

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements for the degree of Doctor of Philosophy in Psychology + Neuroscience

Guelph, Ontario, Canada

© Anna Phan, July, 2013
This thesis examines the rapid, non-genomic effects of estrogens on neural plasticity and learning. Estrogens are classically known to affect gene transcription events, however they have more recently been found to also rapidly activate second messenger systems within 1 hr of administration. Therefore, we first examined the rapid effects of 17β-estradiol, and an estrogen receptor (ER) α and ERβ agonist on three different learning paradigms: object placement, object recognition, and social recognition. We found that both systemic injections and intrahippocampal delivery of 17β-estradiol and the ERα agonist improved performance on all 3 learning paradigms within 40 min of hormone administration. However, the ERβ agonist administered systemically or intrahippocampally, improved performance only on the object placement learning paradigm, while having no effect on object recognition, and impairing social recognition at high doses. To elucidate how estrogens might rapidly affect learning, we examined how estrogens rapidly affect the neural plasticity of CA1 hippocampal neurons. We found that 17β-estradiol and the ERα agonist increased dendritic spine density in CA1 hippocampal neurons within 40 min of administration, suggesting that estrogens rapidly increase the density of synapses within this brain region. Conversely, the ERβ
agonist did not affect spine density, or decreased spine density. In addition, by using whole-cell patch clamp recordings of CA1 pyramidal neurons, we were able to determine that 17β-estradiol and the ERα agonist rapidly reduced AMPA receptor (but not NMDA receptor) mediated membrane depolarizations after 15min of hormone application. Similar to above, the ERβ agonist had no effect on AMPA or NMDA receptor mediated membrane depolarizations. These data suggest that estrogens rapidly promote the development of immature synapses (which contain low levels of synaptic AMPA receptors) within the CA1 hippocampus. Immature spines provide synaptic sites at which new memories can be stored and are thought of as “learning spines” (Kasai et al, 2003). Therefore, estrogens (through ERα) may rapidly induce the formation of hippocampal immature spines to promote learning.
Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Elena Choleris, for her patience, kindness, guidance and encouragement. It was due to her incredible ability to trust in her students that I was able to grow as an independent researcher during the last 6 years, and through her generosity that I was able to participate in deciding the direction of my research project. Thank you, Elena, for allowing me the freedom to make my own mistakes and pursue my passion for research! It has been a wild ride!

I would also like to thank my co-advisor, Dr. Neil MacLusky, and committee member Dr. Boyer Winters for their guidance, time, and invaluable input over the course of my project. Thank you very much for all your help.

It is doubtful I would have survived this experience without the support of the wonderful graduate students in Psychology. The Choleris lab graduate students, Amy Clipperon Allen, Christopher Gabor, and Kelsy Ervin, thank you for your support and help over the years. I feel truly lucky to have worked in such a supportive environment. Similarly, to the NACS graduate students, Craig Allen, Erin Cummins, Jeff Franson, Derek Jacklin, AnneMarie Levy, Erin Rock, Martin Sticht, thank you for sharing in all the ups and downs of research life. Perhaps it is inevitable that our bonds of friendship become stronger after such a harrowing ordeal! Thank you all for your love and friendship over the years. I hope that future cohorts will also be lucky enough to have such a great support network.

Lastly, I would like to thank my family for their support and for cheering me along from the sidelines. Thank you for your patience!
# Table of Contents

ABSTRACT .......................................................................................................................... ii
Acknowledgements ................................................................................................................ iv
Table of Contents .................................................................................................................. v
List of Tables ........................................................................................................................ ix
List of Figures ......................................................................................................................... x
List of Abbreviations: .......................................................................................................... xi

CHAPTER 1: General Introduction ....................................................................................... 1
  Estrogens ............................................................................................................................... 2
  Estrogens and Estrogen Receptors ...................................................................................... 4
  Modes of Estrogen Action: Genomic and Non-Genomic Actions ..................................... 5
  Rapid Estrogenic Effects on Behaviour ............................................................................. 7
    Rapid Estrogenic Effects on Anxiety ............................................................................... 9
    Rapid Estrogenic Effects on Aggression ......................................................................... 10
    Rapid Estrogenic Effects on Sexual Behaviour ............................................................. 11
  Rapid Estrogenic Effects on Memory Consolidation ....................................................... 13
    Rapid Estrogenic Effects on Memory Consolidation: Experimental Evidence .......... 14
  The Hippocampus as a Site for Rapid Estrogenic Action on Learning and Memory ....... 16
  Rapid Estrogenic Effects on Neuronal Dendritic Spines ................................................. 18
    Rapid Estrogenic Effects on Neuronal Electrophysiology .......................................... 22
      Electrophysiology Experiments: Mice ....................................................................... 24
      Electrophysiology Experiments: Male Rats ................................................................. 25
      Electrophysiology Experiments: Female Rats ............................................................... 27
  Objectives ........................................................................................................................... 32

CHAPTER 2: Low Doses of 17β-Estradiol Rapidly Improve Learning and Increase

  Hippocampal Dendritic Spines .......................................................................................... 34
  Material and Methods ....................................................................................................... 38
    Subjects ............................................................................................................................ 38
  Ovariectomy Surgery ........................................................................................................ 39
Rapid Learning Paradigms........................................................................................................ 40
Social Recognition Paradigm.................................................................................................. 41
Object Recognition Paradigm.................................................................................................. 42
Object Placement Paradigm..................................................................................................... 42
Behavioural Data Analysis ...................................................................................................... 42
Olfaction Test............................................................................................................................ 43
Dendritic Spine Analysis .......................................................................................................... 43
Statistical Analysis .................................................................................................................. 45
Results ....................................................................................................................................... 45
Learning Paradigms.................................................................................................................. 45
Social Recognition.................................................................................................................... 45
Object Recognition.................................................................................................................. 47
Object Placement..................................................................................................................... 47
Olfaction Test............................................................................................................................. 48
Dendritic Spines......................................................................................................................... 48
Discussion:................................................................................................................................. 49
Rapid effects of 17β-estradiol on learning ............................................................................... 49
Rapid effects of 17β-estradiol on dendritic spines................................................................. 52
Conclusions:.............................................................................................................................. 54

CHAPTER 3: Rapid Effects of Estrogen Receptor α and β Selective Agonists on Learning and Dendritic Spines in Female Mice .................................................................................. 62
Introduction............................................................................................................................... 64
Materials and Methods ............................................................................................................ 67
Subjects..................................................................................................................................... 67
Drugs .......................................................................................................................................... 67
Rapid Learning Paradigms.......................................................................................................... 68
Social Recognition Learning Paradigm ..................................................................................... 69
Object Recognition Learning Paradigm .................................................................................... 69
Object Placement Learning Paradigm ..................................................................................... 70
Olfaction Test............................................................................................................................ 70
Behavioural Data Analysis ...................................................................................................... 70
CHAPTER 4: The Hippocampus Mediates Estrogens’ Rapid Effects on Learning Through ERα ................................................................. 97
  Abstract .................................................................................. 98
  Materials and Methods ............................................................... 102
    Subjects .................................................................................. 102
  Learning Experiments ................................................................. 103
    Surgery: Ovariectomy and Cannulation ....................................... 103
  Rapid Learning Paradigms ............................................................. 104
    Social Recognition Learning Paradigm ......................................... 105
    Object Recognition Learning Paradigm ....................................... 106
    Object Placement Learning Paradigm ......................................... 106
  Behavioural Data Analysis ............................................................ 106
  Electrophysiology Experiments ...................................................... 107
  Results ...................................................................................... 109
  Rapid Learning Paradigms ............................................................. 109
    Intrahippocampal Delivery of 17β-estradiol .................................. 109
    Intrahippocampal Delivery of ERα Agonist PPT ......................... 111
    Intrahippocampal Delivery of ERβ Agonist DPN ......................... 112
    Habituation to Stimuli - Investigation Durations ....................... 113
  Patch-clamp recordings ............................................................... 114
Discussion .......................................................................................................................... 115
Intrahippocampal Learning Experiments ................................................................. 115
Electrophysiology Experiments ............................................................................. 118
Different Roles for ERα and ERβ in Learning and Memory ................................. 119
Estrogens and the Creation of Immature Synapses ............................................. 121
Conclusions .................................................................................................................. 123
Acknowledgements .................................................................................................... 123
CHAPTER 5: General Discussion .............................................................................. 139
New Synapse Formation and Learning and Memory .............................................. 141
Different Mechanisms of Estrogen Action ............................................................. 143
Significance and Applications of Studying Estrogens and Learning ................... 145
CHAPTER 6: References ............................................................................................. 148
List of Tables

Table 1: A list and description of the behaviours for learning paradigms .................... 56
Table 2: Description of behaviours from learning experiments. ................................. 86
Table 3: A list and description of the behaviours for learning paradigms ..................... 124
List of Figures

Figure 1: Steroidogenic pathway .......................................................................................... 3
Figure 2: Hippocampal subregions ....................................................................................... 19
Figure 3: Rapid effects of 17β-estradiol on learning and memory paradigms ................. 57
Figure 4: Images of Golgi-Cox stained hippocampal CA1 neurons ................................ 59
Figure 5: 17β-estradiol effects on dendritic spines in the stratum radiatum .................. 60
Figure 6: 17β-estradiol effects on dendritic spines in the lacunsum-moleculare .......... 61
Figure 7: Time line of events for the difficult and easy behavioural paradigms ............. 87
Figure 8: Images of hippocampus or CA1 hippocampal neurons .................................... 88
Figure 9: Rapid effects of PPT on learning paradigms ....................................................... 89
Figure 10: Rapid effects of PPT on dendritic spine density and length ............................ 91
Figure 11: Rapid effects of DPN on social recognition ..................................................... 92
Figure 12: Rapid effects of DPN on object recognition ..................................................... 93
Figure 13: Rapid effects of DPN on object placement ..................................................... 94
Figure 14: Rapid effects of DPN on dendritic spine density and length .......................... 95
Figure 15: Rapid effects of intrahippocampal 17β-estradiol on learning paradigms ....... 125
Figure 16: Rapid effects of intrahippocampal PPT on learning paradigms ....................... 127
Figure 17: Rapid effects of intrahippocampal DPN on learning paradigms ...................... 129
Figure 18: Rapid effects of 17β-estradiol, PPT and DPN on (S)-AMPA mediated membrane depolarizations in CA1 neurons: traces ............................................... 131
Figure 19: Rapid effects of 17β-estradiol, PPT and DPN on NMDA mediated membrane depolarizations in CA1 neurons: traces ............................................... 133
Figure 20: Rapid effects of 17β-estradiol, PPT and DPN on (S)-AMPA and NMDA mediated membrane depolarizations in CA1 neurons ........................................... 135
Supplemental Figure S1: Chocolate chip olfaction test ..................................................... 96
Supplemental Figure S2: Cannula placements from 17β-estradiol intracranial experiments ......................................................................................................................... 136
Supplemental Figure S3: Cannula placements from ERα agonist PPT intracranial experiments ..................................................................................................................... 137
Supplemental Figure S4: Cannula placements from ERβ agonist DPN intracranial experiments ..................................................................................................................... 138
List of Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARH</td>
<td>arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotropic factor</td>
</tr>
<tr>
<td>CA1</td>
<td>cornu ammonis 1 (hippocampal subregion)</td>
</tr>
<tr>
<td>CA3</td>
<td>cornu ammonis 3 (hippocampal subregion)</td>
</tr>
<tr>
<td>CAMKII</td>
<td>calcium-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CB</td>
<td>endocannabinoid</td>
</tr>
<tr>
<td>CB1</td>
<td>endocannabinoid receptor 1</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPN</td>
<td>diarylpropionitrile</td>
</tr>
<tr>
<td>E2-BSA</td>
<td>17β-estradiol conjugated with bovine serum albumin</td>
</tr>
<tr>
<td>EB</td>
<td>estradiol benzoate</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>ERαKO</td>
<td>estrogen receptor alpha knockout mouse</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GPER</td>
<td>G-protein-coupled estrogen receptor (aka GPR30)</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
</tr>
<tr>
<td>IR</td>
<td>investigation ratio</td>
</tr>
</tbody>
</table>
LTD.........................................................long-term depression
LTP..........................................................long-term potentiation
mEPSC ..................................................miniature excitatory postsynaptic current
mGluR .....................................................metabotropic glutamate receptor
mTOR.....................................................mammalian target of rapamycin (involved in protein synthesis)
NMDA......................................................N-methyl-D-aspartate
PBS........................................................phosphate buffered saline
PI3K ......................................................phosphatidylinositol 3-kinase
PKA........................................................protein kinase A
PKC ........................................................protein kinase C
PKG ........................................................protein kinase G
PPT .......................................................... propyl pyrazole triol
RNA ........................................................ ribonucleic acid
VMH ......................................................ventral medial nucleus of the hypothalamus
WHI........................................................women’s health initiative
WT ........................................................wild type mouse
CHAPTER 1: General Introduction
**Estrogens**

Estrogens are most commonly known for their role in reproduction and related behaviours. However, in addition to their effects on reproduction, estrogens have a surprisingly wide range of action on many other biological systems, including but not limited to, the cardiovascular system, metabolic system, immune system, skeletal system, and the nervous system (reviewed in Choleris, *et al* 2008; Cui, *et al* 2013; Knowlton and Lee 2012; Nilsson, *et al* 2001; Turgeon, *et al* 2006; Wilson, *et al* 2006). Estrogenic action in the brain affects a variety of different behaviours, both reproductive and non-reproductive in nature. A few examples of non-reproductive behaviours that are mediated by estrogens include feeding, anxiety, as well as learning and memory (reviewed in Choleris, *et al* 2008).

Estrogens are a group of steroid hormones that are synthesized from cholesterol through the steroidogenic pathway (Figure 1), of which 17β-estradiol is the most common naturally occurring form (Cui, *et al* 2013; Rodgers 1990). Due to their chemical structure, estrogens are lipid soluble and pass readily through cell membranes. In addition to the gonads, which produce 85-90% of circulating serum estradiol, estrogens are also synthesized *de novo* by several other tissues; the adrenal glands, adipose tissues, and recently, they have also been shown to be produced by brain tissue (Cui, *et al* 2013; Hojo, *et al* 2008; Nelson 2005). Interestingly, the levels of estrogens in brain tissues are not always predicted by the levels of estradiol found in blood serum (Hojo, *et al* 2009; Konkle and McCarthy 2011). Thus the brain appears capable of synthesizing estrogens in a manner that is at least partly independent of gonadal steroid production. These findings have interesting implications and consequences for the role of estrogens in behaviour,
which for the moment, remain poorly understood (Hojo, et al 2009; Konkle and McCarthy 2011).

Figure 1: Steroidogenic pathway (adapted from Steimer 1993).
Estrogens and Estrogen Receptors

Estrogens exert their actions through several receptors. The two classical transcriptional estrogen receptors (ER) described in the 1960’s and mid-1990’s are known today as ERα and ERβ, respectively (Jensen and Jacobson 1962; Kuiper, et al 1996). These two receptors are nuclear transcription factors that arose from an ancient gene duplication event approximately 450 million years ago, and share ~95% sequence similarity in the DNA binding domain and ~55% sequence similarity in the ligand binding domain of the receptor (Kelley and Thackray 1999; Kuiper, et al 1996). Both ERα and ERβ are highly expressed within the nucleus, cytoplasm and on the membranes of cells (reviewed in Cui, et al 2013). More recently, an evolutionarily distinct membrane-associated estrogen receptor was described, known today as the G-protein coupled estrogen receptor, GPER (previously known as GPR30; Carmeci, et al 1997; Prossnitz, et al 2008). In addition, there appears to be at least one more membrane estrogen receptor, currently designated ER-X (Toran-Allerand, et al 2002). At this time, ER-X appears to be highly expressed only during development, and is almost undetectable in adult tissues (Cui, et al 2013). Due to their more recent discovery, little is known about the functions of the GPER and ER-X receptors.

The expressions of ERs are known to differ between tissues. ERα and ERβ are expressed in multiple areas of the rodent brain and with different intensities. For example, ERα is expressed in the amygdala, bed nucleus of the stria terminalis, preoptic area, hypothalamus, cortex, hippocampus, as well as several brainstem nuclei (Cui, et al 2013). ERβ has been detected in all of these areas as well, but with varying levels of intensity different from that of ERα. In addition to these brain areas, ERβ has also been
found in areas of the basal ganglia, thalamus, ventral and anterior tegmental nucleus, and more brainstem nuclei (Cui, et al 2013; Merchenthaler, et al 2004). As a result of differences in receptor expression in brain tissue, their variability in expression intensities in different brain areas, as well as different mechanisms of action, the stimulation of ERα and ERβ can lead to different physiological and behavioural outcomes (reviewed in Choleris, et al 2008). For example, ERα stimulation is very important and necessary for the expression of sexual behaviour, whereas ERβ more subtly affects aspects of sexual behaviour (Kudwa, et al 2005; Ogawa, et al 1998; Ogawa, et al 1999).

**Modes of Estrogen Action: Genomic and Non-Genomic Actions**

Estrogens can mediate their actions through two main pathways: genomic and non-genomic. The genomic pathway is initiated through the binding of estrogens to two estrogen receptors, ERα and ERβ (reviewed in Nilsson, et al 2001). The ER-estrogen complex dimerizes and in the nucleus influences gene transcription either directly by acting as a transcription factor (e.g. at estrogen response element sequences in the promoter region of genes), or indirectly by binding to and altering the function of other transcription factors and co-factors (Nilsson, et al 2001). Over the last several decades, many aspects of estrogens’ transcriptional effects and how they influence physiology and behaviour have been examined. Most of the behavioural studies using estrogens have focused on these long-term genomic effects of estrogens, typically resulting in measurable changes 24-48hrs after estrogen administration (reviewed in Choleris, et al 2008). In contrast, the non-genomic or rapid pathway for estrogen action is initiated by estrogen binding to ERα, ERβ, GPER or ER-X located on the cell membrane or in the
cytoplasm. This leads to the activation of intracellular signaling cascades, which can then have physiological and/or behavioural consequences (Cui, et al 2013; Vasudevan and Pfaff 2008). The outcomes of these rapid or non-genomic estrogen effects are typically seen on the timescale of minutes to a couple of hours (Vasudevan and Pfaff 2008; Woolley 2007). Studies into these non-genomic effects of estrogens on physiology and behaviour have developed more recently, approximately within the last 15yrs. For this reason, much less is known about these rapid effects than the genomic effects of estrogens, and will be the focus of the investigations herein. Of importance to note is that these two pathways are not isolated from one another; estrogen initiated signaling cascades can go on to affect transcription, and estrogen induced transcription likely affects the proteins available within the cell to affect signaling cascades (Cui, et al 2013; Vasudevan and Pfaff 2008).

Estrogens have been shown to activate various signaling cascades, likely depending on the cell type, cell populations within tissues (e.g., neural populations), and between species (reviewed in (Carreau, et al 2011; Kelly and Ronnekleiv 2012; Knowlton and Lee 2012; Vasudevan and Pfaff 2008); Estrogenic effects on specific cell signaling cascades as it relates to the experiments performed in this thesis are discussed below, in the section “The hippocampus as a site for rapid estrogen action on learning and memory.”) While it is generally believed that these second messenger systems are initiated through estrogens binding to their estrogen receptors (ERα, ERβ, GPER and ER-X; Cui, et al 2013; Kelly and Ronnekleiv 2012), several investigations have presented evidence suggesting that estrogen molecules directly activate some signaling cascades (e.g., cyclic nucleotide second messenger systems; Szego and Davis 1967;
Weissman, *et al* 1975), or directly affect ion channels (e.g., L-type Ca\(^{2+}\) channel; Sarkar, *et al* 2008; Wu, *et al* 2005). Be that as it may, estrogen receptor selective agonists for ER\(\alpha\) and ER\(\beta\) both lead to non-genomic effects on signaling cascades as well as behaviour, often in differing ways such that the rapid effects of an ER\(\alpha\) agonist are often different from those of an ER\(\beta\) agonist (e.g., Phan, *et al* 2011). This suggests that the estrogen receptors are differentially involved in mediating estrogens’ non-genomic effects. In support of this conventional view that non-genomic effects of estrogens are exerted through estrogen receptor activation (Kelly and Ronneklev 2012), a non-competitive estrogen receptor antagonist (ICI 182780) was recently shown to inhibit rapid estrogenic effects on sexual behaviour and estrogenic effects on memory consolidation (Fernandez, *et al* 2008; Seredynski, *et al* 2013). ICI 182780 was originally designed to prevent estrogens’ transcriptional effects, and antagonizes ER\(\alpha\) and ER\(\beta\) through preventing their ability to form dimers (Curran and Wiseman 2001). For this reason, it appears as though estrogen receptors (ER\(\alpha\) and ER\(\beta\)) and their dimerization may be necessary for some of estrogens’ non-genomic effects in neurons. At the moment, it remains unclear exactly how and through what mechanisms estrogen receptors are able to initiate intracellular signaling cascades (Kelly and Ronneklev 2012) to ultimately affect physiology and/or behaviour.

**Rapid Estrogenic Effects on Behaviour**

Estrogens have been shown to rapidly affect a variety of behaviours. Most of these experiments have been conducted in rodents, although for certain behaviours, especially sexual behaviours, evidence from a wide range of species is available (e.g.,
non-rodent mammals, birds, and fish). This thesis will focus on rodent data since this is most relevant for the experiments presented herein. In general, there are relatively few behavioural studies examining estrogens’ rapid effects, which will be discussed in detail in the following sections. Nevertheless, based on the studies available to date, we may make a few generalizations.

1. The genomic and rapid effects of estrogens do not always cause the same behavioural outcome. This is currently most evident in their effects on aggression in male beach mice and California mice, in which estrogen action through a genomic pathway reduces aggression, while their action through a non-genomic pathway increases aggression (Trainor, et al 2008; Trainor, et al 2007).

2. Different estrogen receptors can have different roles for a given behaviour, depending on whether they act through genomic or rapid pathways. For example, activation of ERα and ERβ has been shown to genomically affect anxiety in rodents, whereas these receptors do not mediate estrogens’ non-genomic effects on anxiety (Kastenberger, et al 2012; reviewed in Choleris, et al 2008).

3. Lastly, there is remarkable consistency in the time required for estrogens to rapidly affect behaviours, both across different behaviours and across different vertebrate species (fish (Lord, et al 2009; Remage-Healey and Bass 2004; Remage-Healey and Bass 2007), birds (Cornil, et al 2006a; Cornil, et al 2006b) and mammals, see below sections). Behavioural
changes become apparent 10-15min after estrogen administration and continue for 20-30min thereafter. While the specific signalling cascades activated through the non-genomic effects of estrogens may differ between brain areas even within the same species (reviewed in McEwen and Alves 1999; Vasudevan and Pfaff 2008), perhaps the consistency with which we see behavioural effects across vertebrate species indicates a conserved mechanism through which estrogens initiate these non-genomic effects, or how the activation of different signalling cascades are able to influence behaviour.

*Rapid Estrogenic Effects on Anxiety*

17β-estradiol rapidly increases anxiety-like behaviour within 2 hours of administration, and this appears to be mediated through GPER in both female and male mice. Treatment with 25µg/kg of 17β-estradiol (but not 250µg/kg 17β-estradiol) decreased the time female and male mice spent in the open arms of an elevated plus maze, the light compartment in a light-dark choice test, and the center of an open field, behaviours which are all indicative of increased anxiety in these subjects (Kastenberger, et al 2012). In females, the ERα agonist PPT and ERβ agonist DPN did not have a rapid effect on these measures of anxiety (gonadally intact males were not tested), while the GPER agonist, G-1, replicated the effects of 17β-estradiol in both sexes (Kastenberger, et al 2012). Therefore it appears as though estrogens acting through GPER rapidly increase anxiety in female and male mice. Literature on the genomic effects of estrogens on anxiety is mixed, with some reporting anxiolytic effects while others report anxiogenic
effects, which may depend on estrogen action through ERβ or ERα, respectively (Choleris, et al 2008). Taken together, these studies suggest that different estrogen receptors play different roles in mediating estrogens’ effects on anxiety, depending on whether estrogens are acting through genomic or non-genomic mechanisms.

**Rapid Estrogenic Effects on Aggression**

Estrogens appear to rapidly increase agonistic or aggressive behaviours in several rodent species. In male golden hamsters (*Mesocricetus auratus*), infusion of 10μM of 17β-estradiol into the anterior hypothalamus increased agonistic behaviour (flank marking) in an intruder test within 5min of administration (although it did not directly increase attacks or bites in this species; Haydenhixson and Ferris 1991). In male hamsters, the rapid effects of estradiol appear consistent with their genomic effects, since daily injections of estradiol benzoate over 1-4wks restores aggressive behaviours in castrated males (Grelk, et al 1974).

Aggression in male beach mice (*Peromyscus polionotus*) and male California mice (*Peromyscus californicus*), unlike the hamster, is affected by photoperiod. Both *Peromyscus* species display increased aggression in short, winter-like days compared to long, summer-like days. Male beach mice and California mice housed under short day light conditions administered 75μg/kg or 100μg/kg 17β-estradiol rapidly increased bite frequencies and decreased attack latencies (respectively) in an intruder test within 15min of administration. This was mimicked by the administration of either ERα or ERβ agonists (Trainor, et al 2008; Trainor, et al 2007). The genomic actions of 17β-estradiol, and ERα or ERβ agonists under these short day light conditions also increased aggressive
behaviours (Trainor, et al 2007). Interestingly, when similar experiments were performed in animals that were housed in long day light cycles, neither estradiol nor ER agonists non-genomically affected aggressive behaviours (Trainor, et al 2008; Trainor, et al 2007). However, under long day light conditions, 17β-estradiol, ERα or ERβ agonist administration to male beach mice for 12 days prior to testing appears to be involved in decreasing aggressive behaviours, presumably through genomic mechanisms (Trainor, et al 2007). These differences in estrogens’ ability to affect aggression are also not due to seasonal changes in hormone levels (Trainor, et al 2008; Trainor, et al 2007). Therefore, in male beach and California mice, estradiol can rapidly enhance aggressive behaviours or inhibit aggressive behaviours depending on their rapid or genomic mechanisms of action which interacts with photopheriod (Trainor, et al 2008; Trainor, et al 2007). For estradiol’s effects on aggression, it appears as though activation of ERα or ERβ play similar roles, as both rapidly increased aggressive behaviours during short days, and genomically decreased aggression during long days (Trainor, et al 2007).

**Rapid Estrogenic Effects on Sexual Behaviour**

Work with sexual behaviour in female rodents seems to indicate that estrogens are unable to rapidly increase expression of female sexual receptivity *per se*. Rather, estrogens acting through non-genomic mechanisms appear to augment the necessary genomic actions of estrogens for the expression of the lordosis reflex, a measure of female sexual receptivity. The administration of 17β-estradiol conjugated to bovine serum albumin (E2-BSA), which limits the distribution of estrogen to the plasma membrane of cells, to the ventromedial nucleus of the hypothalamus (VMH) was able to activate the lordosis behaviour in female rats when they were tested 2 days later.
(Vasudevan, et al 2005). However, this facilitatory effect of E2-BSA on lordosis behaviour was only present when combined with a second pulse of regular 17β-estradiol 5hrs before or after E2-BSA administration to the VMH. The administration of E2-BSA alone was not sufficient to induce lordosis (Vasudevan, et al 2005). This suggests that while non-genomic actions of 17β-estradiol may augment female sexual behaviour, the genomic actions of estrogens in the VMH is necessary for the expression of lordosis.

The arcuate nucleus of the hypothalamus (ARH) is also involved in the expression of female sexual receptivity, and estradiol is able to rapidly affect several factors in this brain area that correlate with the expression of lordosis. Administration of estradiol benzoate (EB) or the membrane impermeable estradiol E6-biotin, caused the internalization of µ-opioid receptor in the ARH within 30min of administration (Dewing, et al 2007; Dewing, et al 2008), which is necessary for high levels of lordosis expression 30hr later (Micevych, et al 2009). This non-genomic effect of estrogens appear to be mediated at least in part through ERα and through the activation of protein kinase C (Dewing, et al 2007; Dewing, et al 2008). Therefore, like in the VMH, it appears as though estradiol acting within the ARH rapidly facilitates female sexual receptivity, but these effects are apparent only 30hrs after administration.

Unlike in female rodents, estradiol in male rodents seems to non-genomically enhance sexual behaviour per se. 17β-estradiol injections of 20μg/kg and 100μg/kg to male rats increased their expression of sexual behaviour when tested 15min post-injection and remained elevated until 35min post-injection (Cross and Roselli 1999). These male rats displayed an increase in genital sniffs, number of mounts and a decrease in their latency to mount a receptive female. Interestingly, in the same experiment, injections of
2mg/kg or 10mg/kg testosterone failed to rapidly increase male rat sexual behaviour (Cross and Roselli 1999). Similar estrogenic effects were found in mice. 500µg of 17β-estradiol rapidly increased mounting frequency and intromission frequency in male mice, which were tested 10min after a systemic injection (Taziaux, et al 2007). Copulatory behaviour was monitored in these animals for 30min, although effects were present as early as 20min after hormone injection. While these doses of estradiol likely resulted in supraphysiological plasma estrogen levels (Scharfman, et al 2007), this rapid facilitation of male sexual behaviour is still likely to occur naturally in non-hormonally treated animals. The administration of an aromatase inhibitor to male mice just 10min before testing also nearly eliminated male copulatory behaviour (mounts and intromissions) and their chemoinvestigation of females (ano-genital investigation; Taziaux, et al 2007). Therefore the non-genomic effects of endogenous estradiol are necessary for normal male sexual behaviour in rodents (Taziaux, et al 2007). Notably, the administration of 50µg of 17β-estradiol to aromatase knockout mice, which demonstrate severe impairments in sexual behaviour (Honda, et al 1998), increases mounting and intromission in male mice when tested 10min after injection (Taziaux, et al 2007).

**Rapid Estrogenic Effects on Memory Consolidation**

Almost all of the behavioural work examining rapid estrogen effects has been performed on memory consolidation in female rats. Overall, it appears as though estrogens improve memory consolidation on a variety of tasks. Due to the timing of the experimental procedures of these memory paradigms however (4-48hrs), assessment of memory occurs well beyond the point at which transcriptional events are necessary for memory maintenance (discussed below). Hence the mechanism of estrogenic action in
these experiments is likely through estrogens influencing cell signalling cascades that in turn affect the transcription of proteins involved in memory consolidation (Frick 2012). These rapid effects of estradiol on memory are consistent with their genomic effects, which typically report enhancing effects on a range of learning and memory tasks, and generally implicate ERβ more often than ERα in mediating estrogens’ positive memory effects (reviewed in Choleris, et al 2008).

*Rapid Estrogenic Effects on Memory Consolidation: Experimental Evidence*

Estrogens enhance spatial and object memory consolidation in female rats and mice. When 5-400µg/kg of estrogens (17β-estradiol, estradiol benzoate, or cyclodextrin encapsulated 17β-estradiol) were administered 30min before (Luine, et al 2003), or immediately after learning acquisition on the Morris water maze, object placement or object recognition paradigms followed by a memory retention test 4-48hr later, performance on these tasks was improved (Fan, et al 2010; Fernandez, et al 2008; Frye and Rhodes 2002; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997a; Packard and Teather 1997b; Walf, et al 2008). An ERβ agonist administered immediately after learning acquisition in these experiments was found to facilitate both object recognition and placement memory in female mice (an ERα agonist was not tested) (Walf, et al 2008). Therefore, ERβ may act rapidly to enhance memory consolidation. These effects of estrogens appear to be rapid, since this facilitation of memory consolidation does not occur when the hormone is administered 45-2hrs after learning acquisition (Frye, et al 2007; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997b; Walf, et al 2006).
The timeframe for memory assessment (4-48hrs after learning acquisition) and estrogen treatment (immediately post-acquisition) in these studies support a role for estrogens in memory consolidation, during a time when new memories are transcription dependent. Inhibition of transcription or protein synthesis after learning acquisition does not immediately affect memory (short-term memory), but impairs memory when animals are tested between 1-3hrs (and longer) after the learning event (long-term memory; Bourtchuladze, et al 1994; Da Silva, et al 2008; Nguyen, et al 1994; Schwartz, et al 1971). Short-term memory is therefore maintained by second messenger systems, while long-term memory is supported by transcription and protein synthesis (reviewed in Kandel 2001). In addition, because hormone administration 45min-2hrs following learning acquisition does not improve memory in these experiments, estrogens are unlikely to have facilitated retrieval to produce memory enhancements (Frye, et al 2007; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997b; Walf, et al 2006). Together, these pieces of information suggest that the mechanism of estrogenic action in these memory experiments is likely through activation of cell signalling cascades, that in turn affect the synthesis of proteins necessary for, or that facilitate consolidation of, new memories (reviewed in Frick 2012).

While all the experiments above examine memory that is transcription dependent, one study has examined the effects of estrogens on memory consolidation within 1hr of treatment when memory maintenance may be transcription independent (Bourtchuladze, et al 1994; Da Silva, et al 2008; Nguyen, et al 1994; Schwartz, et al 1971). Male rats (post natal day 18) were tested on a step-down passive avoidance task. Male rats that received an injection of 10µg/kg of EB immediately after training, and tested 1hr later,
demonstrated increased latencies to step down, suggesting memory improvement. However, when administered 23hr before training, there was no facilitation of learning and memory (Xu, et al 2011). Thus these treatments rapidly facilitated memory retention of this task in male rodents. Whether or not estrogens can rapidly affect learning acquisition is unknown.

**The Hippocampus as a Site for Rapid Estrogenic Action on Learning and Memory**

The sites of action in the brain for estrogens effects on behaviour are dependent on the behaviour. For examples, estrogens within the ventral medial hypothalamus are important for sexual behaviours (Vasudevan and Pfaff 2008), estrogens in the anterior hypothalamus affect aggressive behaviours (Trainor, et al 2008; Trainor, et al 2007), while estrogens within the amygdala facilitate social recognition (Spiteri, et al 2010). The non-genomic effects of estrogens have also been shown to affect different intracellular signalling cascades in different brain regions (Vasudevan and Pfaff 2008), which likely explains why estrogens have varied effects on different behaviours. With respect to learning and memory, the most commonly studied structure is the hippocampus. The hippocampus is thought to be necessary for spatial learning and memory but not strictly necessary for other types of learning such as object discrimination or individual recognition (e.g., Squires, et al 2006; Winters, et al 2004). Even still, the hippocampus appears to be able to facilitate non-spatial learning and memory such as object discrimination or individual recognition, especially if testing is performed in the presence of spatial and contextual cues (Phan, et al 2012b). There is some evidence that at least some aspects of the rapid effects of estrogens on learning and memory seem to be mediated through the hippocampus. Intrahippocampal or intracerebroventricular delivery
of 17β-estradiol, cyclodextrin encapsulated 17β-estradiol, or E2-BSA in female rats and mice immediately after learning acquisition replicated estrogens’ rapid systemic effects in facilitating spatial and object memory when tested 24-48hrs later (Fan, et al 2010; Fernandez, et al 2008; Fortress, et al 2013; Lewis, et al 2008; Packard and Teather 1997a; Zhao, et al 2010). These enhancements in memory consolidation appear to occur through estrogens enhancing extracellular signal regulated kinase activity (ERK) in the dorsal hippocampus. ERK signalling is necessary for learning and memory (reviewed in Peng, et al 2010), and experiments demonstrate that estrogen administration increases ERK activity within 5min-1hr (Fan, et al 2010; Fernandez, et al 2008; Lewis, et al 2008). The inhibition of protein kinase A (PKA), NMDA receptors, or phosphatidylinositol 3-kinase (PI3K) have been shown to block the estrogen mediated increase in ERK activation as well as estrogen mediated learning enhancements in object recognition 24-48hrs later (Fan, et al 2010; Lewis, et al 2008, reviewed in Frick 2012). Estrogen induced increases in dorsal hippocampal ERK activation lead to downstream effects, promoting epigenetic changes (histone acetylation and DNA methylation) as well as protein synthesis (through activation of the mammalian target of rapamycin; mTOR), to improve object memory consolidation 24-48hrs after post-learning estrogen administration (Fortress, et al 2013; Zhao, et al 2010, reviewed in Frick 2012). These experiments provide a mechanism through which estrogens within the dorsal hippocampus are able to improve transcription/protein synthesis dependent memory consolidation (reviewed in Frick 2012). That is, they explain how estrogens administered immediately post learning are able to enhance memory consolidation when testing is performed 4-48hrs later (when memory maintenance is transcription/protein synthesis dependent; Bourtchuladze, et al
Whether estrogens’ rapid effects on cell signalling cascades can improve learning or memory independent of transcription and/or protein synthesis remain unclear.

**Rapid Estrogenic Effects on Neuronal Dendritic Spines**

Dendritic spines are small projections that occur along the dendrites of some neurons, first reported by Ramon y Cajal in the 1880’s (Cajal 1888). Today, we recognize them as the postsynaptic sites of the majority of excitatory synaptic connections (reviewed in Alvarez and Sabatini 2007). Estrogens have been shown to rapidly increase dendritic spine densities or synapse density in the CA1 pyramidal neurons of the hippocampus in rats (MacLusky, et al 2005; Mukai, et al 2007; Murakami, et al 2006). Systemic injection of 45 and 60µg/kg 17β-estradiol to female rats or application of 1nM 17β-estradiol onto hippocampal sections from male rats increased dendritic spine density in CA1 neurons (stratum radiatum, stratum oriens and lacunosum-moleculare; Figure 2) when examined 30min or 2h later (respectively; MacLusky, et al 2005; Mukai, et al 2007; Murakami, et al 2006). These increases in spine and synapse density were substantial. On average, these measures increased by as much as 50%. Importantly, electron microscopy of hippocampal tissues revealed that synapse density increased within 30min post systemic 17β-estradiol administration (MacLusky, et al 2005). This implies that reports of non-genomic estrogenic increases in dendritic spine density also have a corresponding structural pre-synaptic input. In support of this argument, a separate experiment using cultured cortical neurons found 10nM of 17β-estradiol significantly increased dendritic spine density within 15min of application (Srivastava, et al 2008).
The authors then conducted an immunostain for synaptophysin, a presynaptic marker, and showed that the majority of spines after estradiol application also had a corresponding presynaptic input (Srivastava, et al 2008). These experiments demonstrate that estradiol can surprisingly increase the synapse density within neural tissues by as much as 50% within 15-30min, implying estradiol is capable of very quickly and significantly altering neural circuitry.

Figure 2: Hippocampal subregions. Modified from Phan, et al 2012. Pyr indicates pyramidal cell layer, rad indicates the stratum radiatum and l-m the lacunosum-moleculare. (A) Nissl stained hippocampus section, scale bar 400µm. (B) Camera lucida drawing of Golgi stained hippocampal primary neuron, scale bar 100µm.

These effects of estradiol on dendritic spines and synapses may be mediated through ERα, rather than through ERβ. The application of PPT has been found to increase spine density within 2hrs of application in CA1 principal neurons in the male rat hippocampus, whereas DPN did not (Mukai, et al 2007; Murakami, et al 2006). Interestingly, in the hippocampal CA3 region in rats, estrogens had the opposite effect and decreased dendritic spine density, which was replicated with the application of PPT.
to hippocampal slices, but not DPN (Tsurugizawa, et al 2005). Furthermore, in cultured
cortical neurons, ERβ activation was shown to be important for spinogenesis (Srivastava,
et al 2010). Therefore, estrogens’ effects on dendritic spines and the estrogen receptor
mediating this effect appear to be heterogenous across different brain regions. Within the
hippocampus, it appears as though the non-genomic estrogen-induced dendritic spine
changes in the hippocampus are mediated through ERα.

Dendritic spine density and morphology are plastic and influenced by sensory
input and/or neuronal signalling (Alvarez and Sabatini 2007). The morphology of
estrogen induced dendritic spines provides information on their functionality. Spines are
typically classified into several functional and morphological types: filopodia, thin
spines, mushroom spines and stubby spines (reviewed in Hering and Sheng 2001). Thin
spines and filopodia are thought to be immature spines, functionally weak with low
numbers of synaptic AMPA receptors, and the sites at which learning can occur
contrast, mushroom, and stubby spines are thought to be mature spines, functionally
strong with an increased number of synaptic AMPA receptors, and the sites at which
memories have been or are currently stored (‘memory spines’; Kasai, et al 2003;
estradiol- and PPT-induced hippocampal spine growth in male rats has revealed that thin
and filopodia type spines are rapidly increased by estrogens (Mukai, et al 2007;
Murakami, et al 2006). Therefore, these results suggest that estrogens promote the
growth of immature spines; sites at which learning can occur (Kasai, et al 2003). These
data are consistent with the observation that estradiol-induced dendritic spines within
cultured cortical neurons are associated with decreased numbers of AMPA receptors on the membrane surface (Srivastava, et al 2008), which is a characteristic of immature spines (Kasai, et al 2003; Matsuzaki, et al 2001; Matsuzaki, et al 2004). Furthermore, consistent with the idea that estradiol-induced spines are ‘learning spines’, the activation of NMDA receptors in estrogen-treated cultured cortical neurons resulted in the enhancement of AMPA receptor mediated transmission in these neurons, above vehicle controls (Srivastava, et al 2008). Therefore overall, estrogens are able to rapidly increase synapse density and theoretically, these new synaptic connections may facilitate learning by providing sites at which new learning events can be stored (reviewed in Srivastava, et al 2013; Srivastava, et al 2011; Srivastava 2012). This is one possible mechanism through which estrogens can lead to enhancements in learning and/or memory.

Intuitively, the theory that estrogens increase “learning spines”, immature synapses that can act as a point of storage for new information, is supported by behavioural experiments demonstrating estrogens rapidly enhance memory consolidation, and vice versa. However, the procedures from behavioural experiments studying non-genomic estrogen-induced memory enhancements are inconsistent with this mechanism of estrogen action. Hormone administration in memory experiments occurs immediately after the learning trial in all (Fan, et al 2010; Fernandez, et al 2008; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997a; Packard and Teather 1997b; Walf, et al 2008; Xu, et al 2011) but one (Luine, et al 2003) behavioural study. If we assumed that systemic administration of 17β-estradiol caused an increase in synapse density 15min after the injection (the earliest timepoint demonstrating an in vitro effect; Srivastava, et al 2008), this would mean at best, spines within the hippocampus
were increased 15min after learning acquisition. As a consequence of this experimental procedure, estrogen-induced dendritic spine or synapse increases are not present at the time of the learning event, and thus there is currently little evidence to support the idea that estrogen-induced spines may facilitate learning.

**Rapid Estrogenic Effects on Neuronal Electrophysiology**

Estrogens have been shown to rapidly affect the electrophysiology of neurons in many different central nervous system areas (e.g., hypothalamus, hippocampus, spinal cord, etc., Vasudevan and Pfaff 2008; Woolley 2007). This section of the thesis will deal with data from the rodent hippocampus, as this is most relevant to the research conducted herein. The experimental evidence outlined within the following section comes mostly from rats, and has been divided based on sex. From these studies, a few generalizations seem to emerge.

1. Results from studies of the non-genomic effects of estrogens on hippocampal electrophysiology are diverse, with reports of estrogens affecting both presynaptic and postsynaptic neurons, and through a variety of different mechanisms that are not mutually exclusive. In general 17\(\beta\)-estradiol seems to consistently enhance the excitability of hippocampal neurons, especially within the CA1 subregion, regardless of the specific mechanisms through which estrogens may act.

2. There appears to be a sex difference in the effective doses of 17\(\beta\)-estradiol for their non-genomic effects in hippocampal sections. Experiments with male sections tend to report electrophysiological effects of 17\(\beta\)-estradiol at
concentrations of 100pM-1nM, whereas for female sections, concentrations of 1nM-100nM appear to be necessary. Based on this observation, estrogens may be able to produce non-genomic effects at much lower doses in male rats compared to female rats.

3. Similar to the results from behavioural studies, estrogens’ genomic and rapid effects on neural electrophysiology are not necessarily the same. While estrogen action through both pathways seem to cause an increase in the excitability of hippocampal neurons, overall the genomic mechanisms seem to implicate NMDA receptors in estrogens electrophysiological effects (e.g., Jelks, et al 2007; Lewis, et al 2008; Smith and McMahon 2005; Smith and McMahon 2006; Smith, et al 2009; Snyder, et al 2011). In contrast, on the whole non-genomic effects of estrogens appear to act on non-NMDA glutamate receptors (i.e., AMPA and kainate receptors), on presynaptic neurons, or interneurons.

4. The non-genomic (and genomic) pathway of estrogen action appear to heterogeneously affect intracellular signalling and the electrophysiology of different CNS tissues (reviewed in Vasudevan and Pfaff 2008). Thus the effects of estrogens within the hippocampus are not necessarily the same as those on hypothalamic neurons, for example. These observations are consistent with estrogens ability to variably regulate different behaviours, likely through their actions on different brain regions.
On the surface, these non-genomic effects of estrogens on hippocampal neurons appear to be consistent with the behavioural reports on estrogens rapidly improving memory consolidation. However, to my knowledge there is no evidence that draws strong parallels between these two rather independent lines of investigation. The doses of 17β-estradiol administered in systemic and intracranial memory consolidation studies (5-400ug/kg and 5-10ug/mouse, respectively; see above sections for details), and to hippocampal sections (100pM-100nM; see “Electrophysiology Experiments” sections below) are difficult to compare. Thus it is possible that doses producing behavioural effects are inconsistent with doses that produce electrophysiological effects. Furthermore, many electrophysiological experiments report enhancements in neurotransmission in response to the stimulation of presynaptic neurons (Foy, et al 1999; Foy and Teyler 1983; Fugger, et al 2001; Kim, et al 2002; Kim, et al 2006; Kim, et al 2006; Kramar, et al 2009; Smejkalova and Woolley 2010; Teyler, et al 1980). If the presynaptic stimulation of neurons is to model incoming signals into the hippocampus, which then increases in the presence of estrogens, the implications of these electrophysiological experiments are that estrogens might enhance learning acquisition. However, in all behavioural experiments (Fan, et al 2010; Fernandez, et al 2008; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997a; Packard and Teather 1997b; Walf, et al 2008; Xu, et al 2011) except one (Luine, et al 2003), estrogens are not on board at the time of learning. Thus, there is little evidence to support the idea that these estrogen-induced changes in hippocampal electrophysiology can lead to learning and memory enhancements.

Electrophysiology Experiments: Mice
Compared with the literature on male and female rats (discussed below), there is surprisingly little electrophysiological data on non-genomic estrogenic effects in mice. One paper has examined the application of 100pM of 17β-estradiol for 15min on hippocampal sections of wildtype (WT) and ERα knock out (ERαKO) mice. Consistent with the majority of data in rats, they found that 17β-estradiol enhanced excitatory postsynaptic potentials (EPSPs) in both male and female WT mice, which was significantly higher than in ERαKO mice of both sexes (Fugger, et al 2001). This seems to suggest that the rapid enhancement in hippocampal EPSPs is mediated through ERα, although it is rather premature to make such a conclusion based on this study alone, since we cannot discount the various problems inherent to the study of transgenic knock out mice, such as developmental effects or compensatory mechanisms (Gingrich and Hen 2000).

*Electrophysiology Experiments: Male Rats*

The first reports of rapid estrogen effects on the electrophysiology of hippocampal neurons came in 1980 by Teyler et. al. They found 100pM of 17β-estradiol applied to hippocampal slices of adult male rats for approximately 20min increased field EPSP (fEPSP) in the CA1 following Schaffer collateral stimulation (Teyler, et al 1980). Interestingly, when they examined female rat hippocampal sections, they found no effects of 17β-estradiol on fEPSPs (Teyler, et al 1980). Subsequently, this effect has been reported multiple times in male rats (Foy, et al 1999; Foy and Teyler 1983; Kim, et al 2002; Kim, et al 2006; Kramar, et al 2009), and it appears to be replicated by the administration of 100nM of ERβ agonist WAY 200070, but not ERα agonist PPT
(Kramar, et al 2009). In addition, this was shown to be limited to the 17β-estradiol isomer (17α-estradiol did not have an effect), and it appeared to increase fEPSPs through enhancing the NMDA receptor mediated currents, as opposed to AMPA receptor mediated currents within 30-145min of hormone application (Foy, et al 1999; Foy and Teyler 1983). However, in contrast to these papers, Kramar et. al., found that the non-genomic 17β-estradiol enhancements of fEPSPs were mediated through the AMPA receptor but not the NMDA receptor (Kramar, et al 2009). Therefore, it appears as though the identity of the receptors mediating this enhancement in fEPSPs in male rat hippocampal sections remains inconclusive. Regardless of the glutamate receptor involved, in agreement with these data, it was found that application of 100pM-1nM of 17β-estradiol to hippocampal sections for 20-30min resulted in an enhancement of long term potentiation (LTP; Foy, et al 1999; Kim, et al 2002; Kramar, et al 2009, but see Mukai, et al 2007), which was replicated with ERβ agonist WAY-200070 (Kramar, et al 2009). Furthermore, estrogen receptor dimerization antagonist ICI 182780 did not prevent estrogens’ rapid enhancement of fEPSPs or LTP (Kramar, et al 2009), suggesting that dimerization of estrogen receptors is not necessary for this effect. These rapid effects of 17β-estradiol do not appear to be confined to the CA1 region of the male hippocampus. It was shown to have the largest potentiating effects on the CA3 associational/commissural afferents (Kim, et al 2006). Therefore, this group of studies demonstrate that 17β-estradiol at 100pM rapidly enhances fEPSPs in the CA1 and CA3 subfields of the hippocampus of male rats. These results would theoretically predict that 17β-estradiol can rapidly lead to enhanced learning in male rats. One study has shown that estradiol benzoate administration to male rats rapidly improved their performance on
a step-down inhibitory avoidance task within 1hr, although in this study, estrogen administration occurred post-learning acquisition (Xu, et al 2011; details in the “Rapid Estrogenic Effects on Memory: Experimental Evidence” section above).

*Electrophysiology Experiments: Female Rats*

Although initially it was reported by Teyler et al., 1980 that estrogens did not affect female rat hippocampal CA1 fEPSPs, since then numerous studies have reported just that. The reason for this discrepancy seems likely due to the concentration of 17β-estradiol that was used in the earlier studies. For male rat hippocampal sections, 100pM-1nM of 17β-estradiol has consistently been reported to elicit electrophysiological effects (Foy, et al 1999; Foy and Teyler 1983; Kim, et al 2002; Kim, et al 2006; Kramar, et al 2009; Teyler, et al 1980). However in female rat hippocampal neurons (acute slices or dissociated neuronal culture), it appears as though much higher concentrations of 17β-estradiol are necessary; typically in the range of 1-100nM (Gu and Moss 1996; Huang and Woolley 2012; Smejkalova and Woolley 2010; Wong and Moss 1991; Wong and Moss 1992; Wong and Moss 1994). Therefore, it appears as though rapid estrogenic effects on hippocampal neurons require a higher dose of 17β-estradiol in female tissues than in male tissues.

The findings from experiments examining rapid estrogenic changes to female rat hippocampal neurons are not as consistent as the findings from male rats. For clarity and ease of explanation, these experiments have been divided here into two groups; the first group consists of experiments implicating postsynaptic mechanisms and the second group contains experiments implicating other mechanisms. It is important to note that these
rapid estrogenic effects are not mutually exclusive. Overall, regardless of the mechanisms implicated in estrogen action, results from experiments generally indicate that estrogens non-genomically enhance the excitability of CA1 neurons.

The first experiments on female rats reported that administration of 1nM 17β-estradiol to hippocampal slices for 1-2min seemed to cause an increase in the membrane potential, and caused neurons to fire action potentials (whole cell patch clamp recordings; Wong and Moss 1991; Wong and Moss 1992). This appeared specific to the 17β-estradiol isomer, since 17α-estradiol did not elicit the same effects (Wong and Moss 1992). 17β-estradiol was also shown to rapidly potentiate glutamate agonist-induced membrane depolarization, and this was replicated with AMPA, kainate, and quisqualate (which are all non-NMDA ionotropic glutamate receptor agonists), but not with NMDA (Wong and Moss 1992). Therefore, 17β-estradiol seemed to potentiate glutamatergic depolarization through a non-NMDA receptor mediated mechanism, since AMPA, kainate and quisqualate are non-NMDA receptor agonists (Wong and Moss 1991; Wong and Moss 1994). This appears to be supported by the observation that 17β-estradiol did not affect the NMDA receptor channel open properties using single channel recording methods (inside-out and outside-in patch clamp; Wong and Moss 1994). This same study also failed to find an effect of kainate (a non-NMDA glutamate receptor agonist; Wong and Moss 1994). However, if 17β-estradiol’s ability to affect glutamate receptors resulted from activating intracellular signalling cascades, this procedure would be unable to detect it since this methodology removes the membrane from the cell, and therefore access to intracellular machinery as well (Gu and Moss 1996; Wong and Moss 1994). In the latest of this series of experiments, the same group identified the kainate receptor as mediating
rapid estrogenic effects on hippocampal electrophysiology (Gu and Moss 1996). For this study, authors obtained intracellular whole cell recordings in acutely dissociated hippocampal neurons from female and male rats, onto which kainate and other agonists were puffed onto the dendrites. Application of 100nM of 17β-estradiol for 3-5min potentiated the kainate induced currents in 30-40% of neurons, whereas again 17α-estradiol was ineffective. This effect of estrogens also appears to be mediated through activation of a G-protein coupled cAMP signalling mechanism that phosphorylates kainate receptors (Gu and Moss 1996). Currently, there is very little known about the role of kainate receptors in learning and memory. One study however, has found that blocking the kainate receptor impaired short-term memory (within 20min of learning acquisition) but had no effect on long-term memory (24hrs after learning acquisition; Barker, et al 2006). Therefore, estrogens may improve short-term memory through enhancing kainate receptor mediated neurotransmission.

A second group of studies pursuing the rapid effects of estrogens on hippocampal electrophysiology in female rats have found different effects compared to the reports discussed above. The first study is in contrast to earlier reports that 1nM 17β-estradiol did not appear to affect presynaptic vesicular release in the CA1 hippocampus (Gu and Moss 1996; Wong and Moss 1992). As above, they found that 17β-estradiol potentiates excitatory transmission in the hippocampus, however they found that this occurred through a presynaptic mechanism which appears to be mediated by ERβ (Smejkalova and Woolley 2010). Application of 17β-estradiol to adult female rat hippocampal slices for 10-15min caused a potentiation in the CA1 excitatory postsynaptic current (EPSC) evoked by stimulation of the Schaffer collaterals (Smejkalova and Woolley 2010).
Interestingly, this effect was heterogenous (exhibited a bimodal distribution; estrogens affected one neuronal population, but did not affect another population of neurons), specific to the \(17\beta\)-estradiol isomer (\(17\alpha\)-estradiol had no effect), and the highest dose of \(17\beta\)-estradiol tested was most effective (doses range: 100pM-100nM). Furthermore they showed that \(17\beta\)-estradiol within 15min increased the amplitude of paired-pulse currents, and a corresponding decrease in paired-pulse ratios (amplitude of second pulse divided by the first pulse), which was the result of a larger increase in the amplitude of the first paired-pulse (Smejkalova and Woolley 2010). Thus the authors demonstrated that \(17\beta\)-estradiol seems to increase hippocampal excitability by increasing vesicular release probabilities of the Schaffer collaterals. This effect was replicated by the ER\(\beta\) agonist DPN, but not ER\(\alpha\) agonist PPT or GPER agonist G1, and this effect was not blocked by estrogen antagonist ICI 182780, suggesting that dimerization is not necessary for this effect (Smejkalova and Woolley 2010).

In another study, \(17\beta\)-estradiol seemed to reduce inhibitory synaptic transmission in the CA1 hippocampus through an ER\(\alpha\) mechanism (Huang and Woolley 2012). Application of 10nM or 100nM of \(17\beta\)-estradiol, or 200nM of ER\(\alpha\) agonist PPT, for 10min significantly decreased inhibitory postsynaptic currents (IPSCs) measured in CA1 hippocampal neurons in approximately 50% of neurons (Huang and Woolley 2012). Interestingly, these effects were only found to occur in female rat hippocampal sections; no effects of estradiol in IPSCs were found to occur in male hippocampal sections (Huang and Woolley 2012; Kramar, et al 2009). Furthermore, induction of this effect of \(17\beta\)-estradiol appears to be dependent on the metabotropic glutamate receptor (mGluR1), since an mGluR1 antagonist abolished the attenuation of IPSCs (Huang and Woolley
2012). While the endocannabinoid (CB) receptor (CB1) also seems important for this effect (determined using a CB1 antagonist and agonist), 17β-estradiol was still able to attenuate IPSC amplitudes in gamma-Aminobutyric acid (GABA) neurons that were not responsive to CB1 antagonists, albeit the proportion of these neurons was much smaller (Huang and Woolley 2012). Thus, it appears as though estradiol-ERα-mGluR1 interactions on interneurons lead to attenuation of CA1 hippocampal IPSCs, and that the majority of these interactions likely result in cannabinoid-mediated CB1 induced decreases in GABA release (Huang and Woolley 2012). The impact of this non-genomic estrogenic effect on learning and memory is less clear. While the CB system has been implicated in learning and memory, currently it appears to be important only for extinction, and not learning acquisition (Marsicano and Lafenetre 2009). Therefore, if one accepts the most parsimonious interpretation of these non-genomic estrogenic effects (that is, they mediate changes through the CB system; Smejkalova and Woolley 2010), the role of these findings for directly enhancing learning and/or memory becomes less clear.

Nevertheless, taken together, these last two studies very elegantly indicate a division of function of the ERα and ERβ receptors: ERα receptors on GABAergic interneurons reduce inhibitory neurotransmitter release, while simultaneously, ERβ induces an increase in presynaptic vesicular release by the CA3 neurons. Non-genomic actions at both receptors may work in parallel to enhance excitatory neurotransmission within the CA1 hippocampus in female rats. These effects of estradiol on hippocampal neurotransmission can theoretically lead to enhanced learning and memory effects, and provide a possible mechanism for 17β-estradiol’s non-genomic enhancement of memory
in female rats, as discussed in the above “Rapid Estrogenic Effects on Memory: Experimental Evidence” section. Although for memory experiments, it is important to note that estradiol administration typically occurred after learning acquisition (Fan, et al 2010; Fernandez, et al 2008; Frye and Rhodes 2002; Gresack and Frick 2006; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997a; Packard and Teather 1997b; Walf, et al 2008; Xu, et al 2011). Therefore the increases in hippocampal excitatory neurotransmission likely did not occur in these behavioural learning experiments until approximately 10-15min following learning acquisition. Thus, if increased hippocampal excitability is the mechanism through which estrogens rapidly enhanced memory, it must have done so via enhancing memory consolidation. Currently there is little evidence supporting the ability of estrogens to rapidly enhance learning acquisition (Luine, et al 2003).

**Objectives**

The aims of this thesis were to determine whether estrogens could affect learning within a non-genomic time frame in a manner consistent with their effects on other behaviours such as sexual behaviour, aggression and anxiety (Chapter 2). This required the development of learning paradigms (or modification of existing learning paradigms) such that they could be completed within a rapid timeframe (under 1hr) to suite our experimental question. Following this, we attempted to elucidate the mechanisms through which estrogens affect learning. We first started with whether these effects were mediated through ERα or ERβ (Chapter 3). We then hypothesized that any non-genomic learning effects of estrogens would occur through estrogenic changes in cell signalling or neural plasticity within neurons. In order to investigate this, we first needed to determine
the site of action for estrogens’ effects on learning, and chose to focus on the
hippocampus (Chapter 4). Estrogens non-genomic effects on two aspects of neural
plasticity were examined within the hippocampus, dendritic spine density (Chapter 2 and
3) and glutamate neurotransmission (Chapter 4).
CHAPTER 2: Low Doses of 17β-Estradiol Rapidly Improve Learning and Increase Hippocampal Dendritic Spines

Abstract

While a great deal of research has been performed on the long-term genomic actions of estrogens, their rapid effects and implications for learning and memory are less well characterized. The often conflicting results of investigations on estrogenic effects on learning and memory may be due to complex and little understood interactions between genomic and rapid effects. Here we investigated the effects of low, physiologically relevant, doses of 17β-estradiol on three different learning paradigms that assess social and non-social aspects of recognition memory and spatial memory, during a transcription independent period of memory maintenance. Ovariectomized female CD1 mice were subcutaneously administered vehicle, 1.5 µg/kg, 2 µg/kg, or 3 µg/kg of 17β-estradiol 15 minutes prior to social recognition, object recognition or object placement learning. These paradigms were designed to allow the testing of learning effects within 40 min of hormone administration. In addition, using a different set of ovariectomized mice, we examined the rapid effects of 1.5 µg/kg, 2 µg/kg, or 3 µg/kg of 17β-estradiol on CA1 hippocampal dendritic spines. All 17β-estradiol doses tested impacted learning, memory, and CA1 hippocampal spines. 17β-estradiol improved both social and object recognition, and may facilitate object placement learning and memory. In addition 17β-estradiol increased dendritic spine density in the stratum radiatum subregion of the CA1 hippocampus, but did not affect dendritic spines in the lacunosum-moleculare, within 40 min of administration. These results demonstrate that the rapid actions of 17β-estradiol have important implications for general learning and memory processes that are not specific for a particular type of learning paradigm. These effects may be mediated by the rapid formation of new dendritic spines in the hippocampus.
Introduction

Estrogens have a variety of effects on the central nervous system, which typically require hours or even days to take effect (see Nilsson, et al 2001) and are likely mediated through transcriptionally regulated changes in gene expression. Recently, it has been shown that estrogens affect cell signalling molecules, neuronal excitability, and behaviour as rapidly as 15min after administration (reviewed in Woolley 2007; Vasudevan and Pfaff 2008). These rapid effects are less well characterized than the genomic effects, but they may combine to produce complex results, which may help explain how estrogens often yield conflicting results for learning and memory ranging from impairment to improvement (reviewed in Choleris, et al 2008). However, our knowledge about the implications of estrogenic rapid actions for learning and memory is still very limited.

Estrogens affect some behaviours as little as 15-35min after administration, a timing consistent with their non-genomic effects, on neuronal electrophysiology and cell signalling cascades. 17β-estradiol administered to males of several species increased sexual behaviour and aggression within this short time interval (Cross and Roselli 1999; Cornil, et al 2006a; Trainor, et al 2008; Charlier, et al 2010). A few studies attempted to examine estrogen's rapid effects on memory consolidation. Administration of 17β- and 17α-estradiol immediately (but not 45min or 2hrs) after learning acquisition in an object recognition and spatial learning task improved performance 4-48h later, supporting a facilitatory role for estrogens on memory consolidation (Luine, et al 2003; Walf, et al 2008; Inagaki, et al 2010; Gresack and Frick 2006; Fernandez, et al 2008; Fan, et al 2010).
Recently, we demonstrated for the first time that estrogen receptor (ER) $\alpha$ and ER$\beta$ agonists rapidly affect learning acquisition and hippocampal dendritic spine density within 40min of systemic administration (Phan, et al 2011). Activation of ER$\alpha$ enhanced social recognition, object recognition, and object placement learning. This was consistent with increases in dendritic spine density in the CA1 area of the hippocampus in mice that had not been tested on the learning protocols (Phan, et al 2011). The selective ER$\beta$ agonist facilitated object placement learning and impaired social recognition at higher doses, but failed to improve or impair object recognition learning, and either failed to affect CA1 spine density or decreased it (Phan, et al 2011). However, since behaviours mediated by ER$\alpha$ and ER$\beta$ vary widely (Phan, et al 2011; Choleris, et al 2008), it is not clear whether 17$\beta$-estradiol, which binds equally to ER$\alpha$ and ER$\beta$, would affect learning within the same rapid time frame.

In this study, we used 3 different spontaneous learning paradigms to examine the rapid effects of 17$\beta$-estradiol. We previously used these paradigms to study the rapid effects of ER$\alpha$ and ER$\beta$ agonists (Phan, et al 2011). They do not require extensive training and can be completed 15-40min after hormone treatment. Using these learning paradigms, we examine the rapid effects of estradiol when memories are transcriptionally independent (Nguyen, et al 1994; Bourtchuladze, et al 1994; Da Silva, et al 2008). They also allow us to assess estrogen’s effects on three different learning systems for which their underlying neuroanatomical mechanisms are not completely independent (Dere, et al 2007; Broadbent, et al 2004; Petrulis 2009). We used low dosages of 17$\beta$-estradiol, 1.5$\mu$g/kg, 2$\mu$g/kg, and 3$\mu$g/kg within a physiological range, since rapid estrogen effects are frequently reported for doses higher than required for their genomic effects.
questioning whether these effects are biologically meaningful (e.g. 100 fold higher in some electrophysiology experiments; reviewed in Woolley 2007). The range of doses we used was chosen to be physiologically relevant (Iizuka, et al 1998; Scharfman, et al 2007). The highest dose, 3µg/kg, produced levels of plasma estradiol in ovariectomized rats corresponding to proestrus levels when estrogens naturally peak (Scharfman, et al 2007). Estrogen treatment also increases hippocampal dendritic spine density as quickly as 15-30min after hormone application (MacLusky, et al 2005; Mukai, et al 2007; Murakami, et al 2006; Srivastava, et al 2008). However, to the best of our knowledge, it is not known whether rapidly induced spine changes correspond with any behavioural learning and memory effects. Moreover, since ERα and ERβ agonists rapidly affect mouse hippocampal dendritic spines in opposing directions (Phan, et al 2011), how 17β-estradiol will affect mouse hippocampal spines at this time scale is uncertain. Therefore, in addition to 17β-estradiol’s rapid effects on learning and memory, we also analyzed their rapid effects on dendritic spines within the CA1 area of the hippocampus.

**Material and Methods**

**Subjects**

185 female CD1 mice (*Mus musculus*) were purchased (2 months old; Charles River, Saint-Constant, QC, Canada) and ovariectomized. 9 animals were randomly chosen as stimulus animals for social recognition experiments, 138 subjects were tested in behavioural learning paradigms, 18 subjects were tested for olfaction, and 20 mice were used to investigate 17β-estradiol effects on hippocampal dendritic spines. Mice were housed on a reversed light/dark cycle (12:12h, lights on at 2000h) at 21±1°C.
Subjects were held in clear polyethylene cages (26cm x 16cm x 12cm) with corncob bedding, environmental enrichment (paper nesting material and paper cup), and rodent chow (14% Protein Rodent Maintenance Diet, Harlan Teklad, WI) and tap water \textit{ad libitum}. Experimental mice were individually housed and cages were not cleaned for at least 3 days prior to testing to establish a home cage territory. Stimulus mice were group housed. All behavioural paradigms were conducted in home cage under red light, during the dark phase of their light cycle.

The evening before testing, experimental mice were moved into the testing room to acclimatize, body weights and vaginal smears were taken. To ensure effective ovariectomies, vaginal cells were stained with Giemsa (Sigma-Aldrich, Oakville, ON, Canada). Cycling animals were removed from the data set (1 animal). Test mice were only used in one experiment. Conducted in accordance with the Canadian Council on Animal Care and approved by University of Guelph’s Animal Care and Use Committee.

\textit{Ovariectomy Surgery}

All mice were ovariectomized as previously described in (Clipperton Allen, et al 2011). Briefly, mice were anaesthetized using isoflurane, subcutaneously (s.c.) injected with an analgesic and anti-inflammatory (50mg/kg Rimadyl, Pfizer Canada Inc, Kirkland, QC, Canada). One dorsal incision was made in the skin, then ovaries were removed through two incisions in the dorsal muscles. A surgical clip (9mm wound clips, MikRon Precision Inc., Gardena, CA) was used to close the incision. Experiments were performed 10-15 days after surgery.
Animals were injected s.c. with 1.5µg/kg, 2µg/kg, or 3µg/kg of 17β-estradiol (Sigma-Aldrich, Oakville, ON) or sesame oil (vehicle control) at a volume of 10mL/kg. The dose range was chosen to mimic levels of estradiol observed in the proestrous (highest dose), estrous, and diestrous phases of the estrous cycle. The lowest dose was half of the highest dose, and a middle dose was included between the two, which is comparable to the range of ER agonists we used previously (Phan, et al 2011). In ovariectomized rats the highest dose, 3µg/kg, resulted in plasma estradiol levels in the proestrus range (Scharfman, et al 2007). Our choice of doses is based on the rat literature (Scharfman, et al 2007) because the mouse literature on this is limited, likely due to the challenges in accurately measuring estradiol in small volumes of plasma (Haisenleder, et al 2011). However, in the classic uterotrophic assay, comparable responsiveness was shown in rats and mice to the same dose (per body weight) of estradiol (Padilla-Banks, et al 2001; Harris, et al 2008; Peano, et al 2009). In addition, s.c., injections of 3µg/kg of 17β-estradiol to ovariectomized mice restore uterine weight to that of sham operated mice (Iizuka, et al 1998). Hence, it is likely that the doses we chose fall within the physiological range in mice as they do in rats (Scharfman, et al 2007). Higher doses of 10µg/kg and above resulted in uterine weight increases beyond the sham operated control mice, and it produced supraphysiological levels of plasma estradiol in ovariectomized rats (Iizuka, et al 1998; MacLusky, et al 2005; Sharfman, et al 2007).

Drug treatments were assigned using a random number generator. The injection site was sealed with superglue (instant Krazy glue, Elmer’s Products Canada, Toronto, ON) to prevent leakage. Mice were injected 15min prior to testing. Each learning
paradigm was completed within 40min of drug administration, targeting 17β-estradiol’s rapid effects. Learning paradigms consisted of 2 habituation sessions and a test, each 5min in duration, separated by 5min intervals (previously described in Phan, et al 2011). These learning paradigms were designed such that vehicle controls do not demonstrate learning, to test for improving effects of 17β-estradiol. When given greater numbers of habituations, vehicle-treated control mice successfully perform these tasks (see Phan, et al 2011).

Habituation and test sessions were recorded under infrared light (8mm Handycam Nightshot, Sony, Cambridge, ON, Canada) for ethological analysis. During all intertest intervals, objects and cylinders used to present stimulus mice (described below) were washed using an odorless detergent and baking soda to remove odor cues. Objects were held in place using Velcro, and were tested to ensure mice showed no preference for one object. Objects used: glass cube (4cm x 4cm x 4cm), stainless steel drain catcher (6cm x 6cm x 1cm), and plastic hairclip (4cm x 3cm x 3cm).

Social Recognition Paradigm

Ovariectomized female CD1 stimulus mice 2.5-4 months old were presented to experimental mice in clear Plexiglas cylinders with perforations at the bottom, to allow passage of olfactory cues (as described in Choleris, et al 2006). During habituations, a test mouse was presented with the same two stimulus mice (e.g., A and B) in consistent positions. During test, one of the two stimulus mice was replaced with a novel mouse (e.g., A and C). The individual replaced was counterbalanced.
Object Recognition Paradigm

During habituation sessions, two different objects were presented to the test mouse in consistent positions, while during test, one of the two objects was replaced with a novel third object. The object that was replaced was counterbalanced, and the two positions of the objects remained consistent throughout the paradigm.

Object Placement Paradigm

Two identical objects were placed in 2 out of 4 possible locations within home cage, and these 2 locations were consistent during habituations. During test, one of the objects was moved 12-14cm to a novel location, directly across from the original placement of the object. The object moved was counterbalanced.

Behavioural Data Analysis

Specific numbers of mice used for behavioural learning paradigms are recorded in figure legends for investigation durations (Figure 3B, D and F). Ten behaviours (Table 1) were collected during the paradigms using The Observer Video Analysis software (Noldus Information Technology, Wageningen, Netherlands) by 3 observers blind to drug treatment.

Investigation behaviour was considered active sniffing of stimuli, nose twitching and within ~1-2mm of the stimulus. Exploiting the natural tendency of mice to investigate novel stimuli or displaced stimuli more than familiar ones, we calculated an investigation percent. \( \text{Percent investigation} = \frac{N}{F+N} \times 100\% \), where N is the time a test mouse spends investigating the novel or displaced stimulus (or during habituations, the stimulus that will be replaced/displaced) and F is the time spent investigating the familiar
stimulus. Investigation percent during habituations typically fluctuates around 50% (chance), while during test, if the experimental mice recognize the novel or displaced stimulus, investigation percent is statistically greater than during habituation (~50%; Phan, et al 2011). Investigation percent at habituation 1 and 2 were averaged to minimize ambiguity and errors that may result due to small random fluctuations in mice investigative behaviour. Animals with total investigation durations of less than 5sec during test (3% of animals) as well as outliers (>2SDs ± mean; 2% of animals) were excluded.

Olfaction Test

Mice were food deprived and weighed the evening before testing, then given 1 Hershey’s chocolate chip (approximately 250-300mg) to familiarize them with the food item. To determine whether learning enhancements could have resulted from changes in olfactory capabilities, mice were injected with vehicle (n=8) or 3µg/kg 17β-estradiol (n=10). Forty minutes after injection, ¼ of a Hershey’s chocolate chip (approximately 70-80mg) was buried in the bedding while the experimenter gently tapped the bedding to draw the mouse away from the burial site. The latency for the mice to find the chocolate chip was recorded.

Dendritic Spine Analysis

Twenty experimentally naïve ovariectomized CD1 females were injected s.c. with 1.5µg/kg, 2µg/kg, 3µg/kg of 17β-estradiol or vehicle (5 animals/group) as described above, returned to home cage, and were euthanized 40min later using CO₂ (as per animal care guidelines). Brain extractions were performed as quickly as possible (1min on
average) to limit CO\(_2\) effects on cytoarchitecture. Methods are described in detail in Phan, et al 2011. Briefly, brains were placed in Golgi-Cox solution (1% potassium dichromate, 0.8% potassium monochromate, 1% mercuric chloride) for 3 weeks in the dark, then in 20% sucrose phosphate buffer (PB)(48 hours at 4\(^\circ\)C). Brains were sectioned using a vibrating microtome (Leica VT1000x, Leica Microsystems, Richmond Hill, ON, Canada) at 200\(\mu\)m. Free floating sections were stored in 6% sucrose PB (24 hours at 4\(^\circ\)C), then processed in 4% paraformaldehyde (15min), 1% NH4OH (15min), 1% Kodak rapid fixative (15-60min). Sections mounted on gelatin-coated slides were air dried (1.5-2 hours) and coverslipped.

Images from the stratum radiatum and lacunosum-moleculare subregions of the CA1 hippocampus (Figure 4) were taken (63x oil objective microscope, Axio Imager D1, captured with AxioCam MRc5 digital camera using AxioVision 4.6 software, Carl Zeiss, Toronto, ON, Canada). An observer blind to treatments analyzed images of 5 neurons from each animal using Image J software (version 1.38x, National Institutes of Health, Bethesda, MD). The sampling region was 30-50% (stratum radiatum) and 80-100% (lacunosum-moleculare) the length of the apical dendrite, from greater than 10\(\mu\)m of dendrite length. Dendritic spine density (spines per 10\(\mu\)m) and spine length (distal tip of spine head to edge of dendrite) was measured from 2 secondary dendrites in each of the stratum radiatum and lacunosum-moleculare of the same neuron. These measures were averaged per subregion for every neuron, then an average value was calculated from the 5 neurons for each mouse.
Statistical Analysis

Investigation percent for habituation 1 and 2 were averaged for analysis. Two-way repeated measures ANOVAs were used to analyze behavioural data, with habituation and test as the repeated measure, and treatment as a between groups factor. To reduce type I errors, specific a priori binary mean comparisons were planned in the statistical model to assess the effects of the change in experimental condition at test (novel stimulus/location). Specifically, within the ANOVA model paired t tests were used to assess differences in preference scores between habituation and test within each treatment group, and one-way ANOVAs and Student-Neuman-Keuls (SNK) post hocs were used to assess differences in preference scores at test between doses. Data for preference scores (expressed as a ratio) were arcsin transformed. Dendritic spine data were analyzed using Kruskal-Wallis ANOVA on ranks, and SNK post hocs. Sigmastat version 3.5 was used for all statistical analyses (Systat Software, Inc., Chicago, IL). Statistical significance was set at $P < 0.05$. For brevity, non-significant values/results are not presented unless meaningful.

Results

Learning Paradigms

Social Recognition

17β-estradiol rapidly improved social recognition (Figure 3A). The ANOVA for percent investigation during test indicated a significant main effect of treatment ($F_{3,38}=3.379$, $p<0.05$). Post hoc analysis indicated that the 3μg/kg 17β-estradiol group
had a significantly higher percent investigation at test compared to vehicle \((q=4.476, df=20, p<0.05)\). Furthermore, investigation percent at test was significantly higher than at habituation for all groups treated with \(17\beta\)-estradiol but not for the vehicle control group (vehicle: \(t=1.13, df=10, \text{N.S.}, 1.5\mu g/kg: t=2.49, df=9, p<0.05, 2\mu g/kg: t=2.63, df=9, p<0.05, 3\mu g/kg: t=5.40, df=10, p<0.001\)). This indicates that all groups administered the steroid hormone were able to demonstrate social recognition, whereas the vehicle treated mice could not.

Total social investigation times (amount of time spent sniffing both stimulus animals) were not significantly different between treatment groups, and mice demonstrated normal habituation to stimulus animals (Figure 3B). Total investigation duration differed significantly across habituations and test \((F_{2,76}=41.161, p<0.001)\), and post hoc tests indicated significant decreases in investigation from habituation 1 to habituation 2 and from habituation 1 to test (all \(q>3.69\), all \(p<0.025\)). In addition, there was a significant interaction for treatment across test number for inactivity \((F_{6,76}=2.80, p<0.05)\) and rearing duration \((F_{6,76}=2.53, p<0.05)\). Post hoc analyses revealed a significant increase in inactivity duration from habituation 1 to 2 in the \(3\mu g/kg \ 17\beta\)-estradiol group \((q=3.54, df=10, p<0.05)\), but no differences between treatment groups. Rearing duration was significantly higher during habituation 1 compared to habituation 2 and test within the \(3\mu g/kg \ 17\beta\)-estradiol group \((q=6.66, df=10, p<0.001, q=6.23, df=10, p<0.001, \text{respectively})\). During habituation 1, the \(3\mu g/kg \ 17\beta\)-estradiol group also had higher rearing durations compared to vehicle \((q=5.17, df=20, p<0.01)\). There were no other effects of \(17\beta\)-estradiol treatment on other behaviours analyzed, suggesting that learning and memory enhancements were not due to changes in overall activity.
Object Recognition

Treatment with 17\(\beta\)-estradiol rapidly improved object recognition in mice (Figure 3C). A one-way ANOVA revealed a significant main effect of treatment for percent investigation during test (\(F_{3,44}=2.93, p<0.05\)). Post hoc analysis revealed that animals treated with 1.5 and 3\(\mu\)g/kg of 17\(\beta\)-estradiol had significantly higher test percent investigation values compared to vehicle animals (1.5\(\mu\)g/kg: \(q=3.09, df=20, p<0.05\), 3\(\mu\)g/kg: \(q=3.86, df=22, p<0.05\)). In addition, investigation percent at test was significantly higher than at habituation for all groups treated with 17\(\beta\)-estradiol, indicating they were all able to successfully perform the task, whereas the vehicle control group could not (vehicle: \(t=1.21, df=9, \text{N.S.} \), 1.5\(\mu\)g/kg: \(t=4.29, df=11, p=0.001\), 2\(\mu\)g/kg: \(t=2.46, df=11, p<0.05\), 3\(\mu\)g/kg: \(t=4.21, df=13, p=0.001\)).

Similar to the results found with social recognition, total object investigation was significantly different across test numbers, indicative of normal habituation of mice to the objects (\(F_{2,88}=45.302, p<0.001\); Figure 3D). Habituation 1 total investigation durations were significantly higher than that of habituation 2 and of test (all \(q>4.19\), all \(p<0.01\)). There was no effect of treatment on any other behaviour analyzed, suggesting 17\(\beta\)-estradiol’s effects on object recognition were not secondary to changes in other behaviours.

Object Placement

17\(\beta\)-estradiol may have rapidly facilitated object placement, since all groups of animals receiving the hormone demonstrated successful discrimination of the displaced object, while the vehicle control did not (Figure 3E). Planned comparisons indicate significantly higher investigation percent values during test compared to habituation in
estradiol treated groups (vehicle: $t=1.99$, $df=12$, N.S., 1.5$\mu$g/kg using Wilcoxon signed rank test: $Z=2.70$, $df=9$, $p<0.01$, 2$\mu$g/kg: $t=4.44$, $df=12$, $p<0.001$, 3$\mu$g/kg: $t=4.46$, $df=10$, $p=0.001$).

While treatment did not affect total object investigation durations, we found that total investigation durations were significantly different across test number ($F_{2,86}=62.18$, $p<0.001$; Figure 3F). Mice habituated to the stimuli, since investigation durations at habituation 1 were significantly higher compared to habituation 2 and compared to test (all $q>4.44$, all $p<0.025$). There was no significant effect of treatment on any other behaviour analyzed, suggesting effects of 17$\beta$-estradiol were specific to object placement performance.

Olfaction Test

Administration of 3$\mu$g/kg of 17$\beta$-estradiol did not rapidly affect the latency of mice to find a buried chocolate chip ($p=0.74$, vehicle 16.5±5.8s, 3$\mu$g/kg 14.3±3.5s, mean±SEM). Thus, 17$\beta$-estradiol learning and memory effects are not secondary to improved olfaction in treated animals.

Dendritic Spines

Hippocampal dendritic spines were rapidly changed with 17$\beta$-estradiol treatment (Figure 5). There was a significant main effect of treatment on spine density in the stratum radiatum (Spine density: $H=12.051$, $df=3$, $p<0.01$). Post hoc analysis revealed estradiol at all doses significantly increased spine density in the stratum radiatum compared to vehicle (1.5$\mu$g/kg: $q=4.46$, $df=8$, $p<0.05$, 2$\mu$g/kg: $q=5.61$, $df=8$, $p<0.05$, 3$\mu$g/kg: $q=5.30$, $df=8$, $p<0.05$; Figure 5A,B). However, there was no effect of 17$\beta$-
estradiol on spine length (Figure 5C,D). Changes in spine density and length in the lacunosum-moleculare were not statistically significant (Figure 6).

Discussion:

*Rapid effects of 17β-estradiol on learning*

Treatment with 17β-estradiol improved social recognition, object recognition, and may facilitate object placement performance 40min after systemic administration (Figure 1A,C and E). 3µg/kg of 17β-estradiol improved social recognition, and 1.5 and 3µg/kg 17β-estradiol improved object recognition learning. For all paradigms tested, groups administered 17β-estradiol at any dose successfully completed the learning paradigms, while vehicle controls did not. These results suggest 17β-estradiol rapidly facilitated learning and memory, and that the improvements seen may be on general learning and memory processes, since effects were not specific to one learning paradigm.

By and large, treatment with 17β-estradiol did not affect other behaviours recorded from these animals or their olfactory capabilities. Thus, the rapid effects of estrogens on learning and memory do not seem to be secondary to changes in other behaviours, such as overall activity. However, because we are testing the rapid effects of 17β-estradiol, we cannot methodologically eliminate the possibility that the rapid effects we observed in the 3 learning paradigms may be state-dependent, since serum estradiol has been shown to remain elevated at least 1-2hrs after systemic injection in ovariectomized rats (Scharfman, *et al* 2007). In addition, because these learning paradigms depend on the response of test animals to novelty, apparent enhancements in performance can also be a product of increased interest in novelty *per se*. The fact that
there were no drug treatment effects on total investigation durations in any learning paradigm tested during habituation 1, when both stimuli were equally novel (Figure 1B,D and F), suggests the effects of estradiol in the three learning paradigms are likely not a result of enhanced interest in novelty. However, we cannot discount the possibility that this may occur at the 35min post hormone administration time point when the test was performed.

These rapid effects of 17β-estradiol are likely mediated by ERα, since we have previously shown that ERα agonist PPT rapidly improved performance in the social recognition, object recognition and object placement paradigms (Phan, et al 2011), in a manner similar to our current results with 17β-estradiol. In contrast, ERβ agonist DPN did not improve social or object recognition, but improved object placement performance only (Phan, et al 2011). However, unlike our studies with the ER agonists, the improved performance seen with 17β-estradiol appears more robust, since effects with ER agonists were very specific to one or two doses (out of 4), while all groups receiving 17β-estradiol here were successful in the 3 learning paradigms, the vehicle treated animals were not. This may indicate that either ERα and ERβ interact to produce a synergistic effect, or that other receptors such as the G-protein coupled estrogen receptor (GPER), or as yet other undefined estrogen receptors are involved in mediating this effect. Indeed, recent experiments from our lab indicate that a GPER agonist, G1, also rapidly improved social recognition, object recognition, and object placement performance (Gabor, et al 2011).

Estrogens rapidly affect several neuronal functions known to be important for learning and memory. For examples, 17β-estradiol rapidly affected glutamate receptor trafficking and transmission in neurons (Srivastava, et al 2008; Zhao and Brinton 2007;
Foy, et al 1999; Smejkalova and Woolley 2010) and increased calcium influx (Zhao and Brinton 2007; Zhao, et al 2005; Wu, et al 2005; Sarkar, et al 2008). Furthermore, or perhaps as a result of these effects, estrogen treatment increased activation of calcium-calmodulin-dependent protein kinase II (CaMKII) (Sawai, et al 2002) and the extracellular signal regulated kinase (ERK) signalling pathway (Fernandez, et al 2008; Fan, et al 2010; Srivastava, et al 2008; Zhao and Brinton 2007; Smejkalova and Woolley 2010; Lewis, et al 2008), both of which are activated by Ca\textsuperscript{2+} and critical for learning and memory and the expression of long-term potentiation (LTP) (Malinow, et al 1989; Thomas and Huganir 2004). Lastly, estrogens rapidly facilitate hippocampal LTP (Foy, et al 1999; Zadran, et al 2009, but see Mukai, et al 2007) or long-term depression (Mukai, et al 2007). While there is a fair amount of evidence for estrogens affecting various signalling pathways and electrophysiological properties in neurons, the exact method through which estrogens are able to do so remains unclear. One possible way through which this may occur is estrogens action at L-type Ca\textsuperscript{2+} channels. Estrogens were found to potentiate neuronal L-type Ca\textsuperscript{2+} channels, leading to an increase in Ca\textsuperscript{2+} influx that apparently leads to activation of the ERK signalling pathway (Wu, et al 2005; Sarkar, et al 2008). Also, estrogens rapidly affect (or exert their effects through) various other signalling pathways, such as protein kinase A (PKA) and phosphotidylinositol 3-kinase pathways (reviewed in Vasudevan and Pfaff 2008; Kelly and Ronnekleiv 2009). Whether these pathways might possibly be activated in a similar manner, that is, through enhanced neurotransmission or ion channel functions, is not well known.
Rapid effects of 17β-estradiol on dendritic spines

Treatment with 1.5, 2 or 3µg/kg of 17β-estradiol increased dendritic spine density in the CA1 stratum radiatum within 40min (Figure 3A,B). However, dendritic spine density within the lacunosum-moleculare region of the CA1 did not significantly differ with treatment (Figure 4A,B), nor did dendritic spine length in either subregion (Figure 3C,D, Figure 4C,D). Previous investigations reporting the genomic effects of estrogens on spine density have found significant increases with 17β-estradiol treatment in both the stratum radiatum and lacunosum-moleculare subregion of the hippocampus (e.g., Gould, et al 1990). One possible explanation for the lack of spine increases in the lacunosum-moleculare in the present study may lay with the timing and differences in hormone administration protocol after ovariectomy. Previous studies examining estrogen’s genomic effects typically administered estradiol twice, at approximately 3 days and 5 days post ovariectomy, and brain tissue extraction was done on day 7 (e.g., Gould, et al 1990), whereas we administered 17β-estradiol only once, 10-15 days post surgery and extracted tissues after 40min. Spine density was affected by the timing of estrogen administration following ovariectomy when treatment is delayed by weeks or months (McLaughlin, et al 2008; Smith, et al 2010). Although the timing of hormone administration used here was delayed only by 1 week compared to previous studies (i.e., 3 days vs. 10 days), this along with differences in hormone administration, may help to explain why we found no estrogen-mediated effects on dendritic spines in the lacunosum-moleculare.

The rapid increase in hippocampal spine density following 17β-estradiol treatment paralleled our estradiol-mediated rapid improvements in learning and memory
paradigms. These effects are also consistent with our previous work using ERα agonist PPT, which also rapidly increased CA1 dendritic spines in a manner that paralleled enhancements in performance on learning paradigms (Phan, et al 2011). Therefore, it is possible that estrogen-mediated increases in connections within brain structures relevant to learning and memory may lead to a facilitation of acquisition. Furthermore, since experience and learning itself changes dendritic spine density and shape (reviewed in Yu and Zuo 2011), it would be interesting to determine whether learning-driven and estradiol-driven dendritic spine changes interact in individuals receiving hormone treatment that are also tested in the learning paradigms. Interestingly, when the genomic effects of estrogens on synapse density was examined in rats that were or were not tested in the Morris water maze, estradiol benzoate increased spine density only in animals that did not undergo the learning paradigm (Frick, et al 2004).

These effects of estrogens on spine density are consistent with other studies that found estradiol rapidly increases synapses or spine density (MacLusky, et al 2005; Mukai, et al 2007; Murakami, et al 2006; Srivastava, et al 2008). Rapid estrogen-mediated increases in spine density are thought to occur through the activation of signalling cascades by estrogens, that then affect molecules important for actin cytoskeleton remodelling and dendritic spine formation (reviewed in Sanchez, et al 2012). In addition, estrogen’s rapid effects on dendritic spines appear to be mediated through ERα in the hippocampus, as an ERα agonist can mimic estradiol’s effects (Phan, et al 2011; Mukai, et al 2007; Murakami, et al 2006). The ERβ agonist DPN, instead did not have an effect on spine density in the stratum radiatum or the lacunosum-moleculare (Phan, et al 2011; Mukai, et al 2007; Murakami, et al 2006), or decreased it at higher
doses in the lacunosum-moleculare (Phan, et al 2011). In cultured cortical neurons, however, treatment with ERβ agonist WAY-200070 increased dendritic spine density within 30-60min (Srivastava, et al 2010). Thus the involvement of ERα and ERβ for mediating estrogen-induced dendritic spine increases may be heterogeneous across different brain structures.

Conclusions:

To our knowledge, this is the first report of 17β-estradiol improving learning within 40min of administration. The timeframe used in this study corresponds to the timeframe for which rapid estrogen effects are reported on neuronal electrophysiology, cell signalling mechanisms, and other behaviours such as aggression and sexual behaviour. The results were also obtained during a time in which memory is reported to be independent of transcription. Thus our results provide support for the hypothesis that 17β-estradiol rapidly enhances learning and/or memory in a non-genomic manner. Moreover, we show that the same timeframe and the same doses of 17β-estradiol which produce improvements in learning tasks also rapidly increase dendritic spine density in the CA1 stratum radiatum. Together with our previous results, 17β-estradiol seems to rapidly improve performance in learning tasks and increase dendritic spine density via ERα (Phan, et al 2011).

Rapid estrogen effects tend to be reported at higher dosages than are needed for their genomic effects, calling into question whether these rapid effects are biologically meaningful (reviewed in Woolley 2007). Here we show that dosages of 17β-estradiol equal to and lower than the dosage that produced proestrous-like high physiological
levels of plasma estradiol in ovariectomized female rats (Scharfman, et al 2007) rapidly improves performance on learning tasks in female mice. Hence, these results suggest that 17β-estradiol is able to act rapidly to enhance learning and/or memory in natural systems. Within an animal however, estrogens activate both rapid and genomic pathways, which likely affect one another. For example, rapid and genomic effects of estrogens produced additive effects to induce the estrogen-dependent behaviour lordosis in female rats (Vasudevan and Pfaff 2008; Vasudevan, et al 2005). Whether this is also true for learning and memory remains to be investigated. Effects of estrogens on learning and memory are complex, with reports of estrogen’s effects ranging from impairing to improving learning and memory tasks (reviewed in Choleris, et al 2008). A better understanding of rapid and genomic effects within different neural structures, as well as activation through specific estrogen receptors, may explain these complex effects on learning and memory systems.
Table 1: A list and description of the behaviours collected and analyzed for learning paradigms

<table>
<thead>
<tr>
<th><strong>Behaviour</strong></th>
<th><strong>Description</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniff Stimulus</td>
<td>Sniff/Investigation of object or mouse stimulus. Nose twitching and within 1-2mm of stimuli.</td>
</tr>
<tr>
<td>Bite Stimulus</td>
<td>Biting object or mouse stimulus cylinders.</td>
</tr>
<tr>
<td>Sit/Climb on Stimulus</td>
<td>Sitting/climbing on object stimulus with all four paws off the cage floor.</td>
</tr>
<tr>
<td>Dig</td>
<td>Movement of forepaws propelling bedding in posterior direction.</td>
</tr>
<tr>
<td>Bury</td>
<td>Movement of forepaws pushing bedding away from body in anterior direction.</td>
</tr>
<tr>
<td>Horizontal Exploration</td>
<td>Walking, non-stimulus sniffing and exploration of cage.</td>
</tr>
<tr>
<td>Rearing</td>
<td>Both forepaws off the cage floor.</td>
</tr>
<tr>
<td>Self Groom</td>
<td>Grooming with forepaws moving over face and body.</td>
</tr>
<tr>
<td>Inactivity</td>
<td>Includes behaviours such as sit, lay down, freeze and sleep.</td>
</tr>
<tr>
<td>Stereotypy</td>
<td>Strange, repetitive behaviours (&gt;3 repetitions), such as jumps, head shakes, lid chews, etc.</td>
</tr>
</tbody>
</table>
Figure 3: Rapid effects of 17β-estradiol on learning and memory paradigms. Bar graphs illustrate investigation percent (grey bars average habituation, black bars test).
investigation percent). Line graphs illustrate total investigation durations (H indicates habituation). A, 3.0µg/kg of 17β-estradiol improved social recognition above vehicle controls, and all groups administered 17β-estradiol successfully demonstrated social recognition. B, Total mouse investigation durations were not affected by 17β-estradiol treatment. C, 1.5 and 3.0µg/kg of 17β-estradiol improved object recognition above vehicle controls, and all groups administered 17β-estradiol successfully demonstrated novel object discrimination. D, Total object investigation durations were unaffected by 17β-estradiol treatment. E, All groups administered 17β-estradiol successfully demonstrated novel object location discrimination, in the object placement paradigm. F, 17β-estradiol treatment did not affect total object investigation durations. Asterisks above black bars indicate a significant difference between habituation and test within treatment group. Mean±SEM. *p<0.05, **p<0.01, ***p<0.001.
Figure 4: Images of Golgi-Cox stained hippocampal CA1 neurons. Pyr, pyramidal cell layer; rad, stratum radiatum; l-m, lacunosum-moleculare. A, Scalebar 200µm. B, Scalebar 100µm. C-F, Images of secondary dendrites from CA1 pyramidal neurons, from the stratum radiatum and lacunosum-moleculare of female mice treated with vehicle, 2.0µg/kg or 3.0µg/kg of 17β-estradiol. Scalebars 5µm.
Figure 5: Rapid effects of 17β-estradiol on dendritic spines in the stratum radiatum of female mice. A and B, Treatment with 1.5, 2.0 or 3.0 µg/kg of 17β-estradiol increased dendritic spine density compared to vehicle. C and D, Treatment with 17β-estradiol did not affect spine length. Asterisks indicate a significant difference between vehicle and 17β-estradiol treatment groups. Mean±SEM. ***p<0.001.
Figure 6: Rapid effects of 17β-estradiol on dendritic spines in the lacunosum-moleculare of female mice. Treatment with 17β-estradiol did not significantly affect dendritic spine density (A and B) or dendritic spine length (C and D). Mean±SEM.
CHAPTER 3: Rapid Effects of Estrogen Receptor $\alpha$ and $\beta$

Selective Agonists on Learning and Dendritic Spines in Female Mice

Abstract

Estrogen receptor (ER) agonists rapidly affect neural plasticity within 1h, suggesting they play a functional role in learning and memory. However, behavioural learning experiments on such a rapid time scale are lacking. Therefore we investigated whether the ERα agonist PPT and ERβ agonist DPN could affect social recognition, object recognition or object placement learning within 40min of drug administration. At the same time, we examined their effects on CA1 hippocampal dendritic spines. Ovariectomized female CD1 mice were administered a range of PPT or DPN doses (0, 30, 50, 75 or 150µg/mouse). PPT at the middle doses improved social recognition, facilitated object recognition and placement at a dose of 75µg, and increased dendritic spine density in the stratum radiatum and lacunosum-moleculare. In contrast, DPN impaired social recognition at higher doses, did not affect object recognition, but slightly facilitated object placement learning at the 75µg dose. DPN did not affect spines in the stratum radiatum, but decreased spine density and increased spine length in the lacunosum-moleculare. This suggests that rapid estrogen-mediated learning enhancements may predominantly be mediated through ERα, while the effects of DPN are weaker and may depend on the learning paradigm. The role of ERα and ERβ in learning and memory may vary depending on the timing of drug administration, as genomic studies often implicate ERβ in enhancing effects on learning and memory. To our knowledge, this is the first report of estrogen’s effects on learning within such a short time frame.
Introduction

Estrogens affect many physiological and behavioural processes including reproduction, feeding, mood, and learning and memory (see Choleris, et al 2008). The classical mechanism of action for intranuclear estrogen receptors (ER), ERα and ERβ, is to regulate transcription of target genes, requiring hours to affect protein expression (reviewed in Nilsson, et al 2001). However, estrogens also have non-genomic actions initiated at the cell membrane that influence cell signalling cascades within minutes (reviewed in Vasudevan and Pfaff 2008). While there are many studies on estrogens’ genomic effects, their rapid, non-genomic effects and the functional behavioural implications thereof are not well understood.

plasticity in a way suggesting they play an important role in learning and memory (reviewed in Charlier, et al 2010).

Both ERα and ERβ were localized to neuronal cell membranes in vivo (Blaustein, et al 1992; Hart, et al 2007; Milner, et al 2001; Milner, et al 2005) and can rapidly affect synaptic plasticity. ERα agonist propyl pyrazole triol (PPT) and ERβ agonist diarylpropionitrile (DPN) both affect cell signalling, synaptic transmission, and LTD in hippocampal sections within 1.5h of application (Mukai, et al 2007; Smejkalova and Woolley 2010; Zhao and Brinton 2007). Rapid estrogen-mediated spinogenesis may occur through ERα in the hippocampus, since PPT increased dendritic spines in rat CA1 hippocampal sections, while DPN produced no effect after 2h (Mukai, et al 2007; Murakami, et al 2006). However, in cultured cortical neurons, ERβ agonist WAY-200070 rapidly increased dendritic spines within 30min of application (Srivastava, et al 2010). Therefore both ERα and ERβ can mediate at least some of estrogens’ rapid effects, and thus may modulate learning and memory functions.

Involvement of estradiol and its receptors in learning and memory have been repeatedly shown (reviewed in Choleris, et al 2008; Choleris, et al 2009) in experiments that typically assess estrogens’ effects hours to days after treatment, a time frame consistent with their genomic mechanisms of action. However, rapid behavioural effects have also been reported. Male Japanese quails, rats, and California mice displayed increased sexual behaviour or aggression 15-35min after systemic 17β-estradiol administration (Charlier, et al 2010; Cornil, et al 2006a; Cross and Roselli 1999; Trainor, et al 2008). Enhanced object recognition and spatial memory consolidation have been observed after 17β-estradiol, 17α-estradiol and a selective ERβ agonist when they are
given immediately (but not 45min or 2h) after learning acquisition, with assessments of learning effects performed 4h, 24h or 48h after drug administration (Fernandez, et al 2008; Frick, et al 2010; Inagaki, et al 2010; Luine, et al 2003; Packard and Teather 1997a; Packard and Teather 1997b; Walf, et al 2008). These studies suggest a role for estrogens in consolidation phases of memory formation. Whether estrogens would affect the acquisition phase of memory in a time frame similar to that of rapid effects observed in neurons or for other behaviours (generally within 1h; Charlier, et al 2010) is unknown. Therefore, we modified standard learning paradigms to be completed in 25 min (between 15min and 40min after drug injection), when memory maintenance is transcription independent (Bourtchuladze, et al 1994; Da Silva, et al 2008; Nguyen, et al 1994). We chose the social recognition, object recognition and object placement paradigms because they are spontaneous (i.e., do not require previous training) and assess different memory systems (social information processing, item recognition, and spatial, respectively), whose underlying neuroanatomical mechanisms do not completely overlap (Broadbent, et al 2004; Dere, et al 2007; Petrulis 2009).

Because both ERα and ERβ can rapidly affect neurons, we tested whether ER selective agonists could rapidly affect learning within 40min of injection. To detect both learning improvements and impairments, we developed “difficult” and “easy” versions of the 3 learning tasks, respectively. The easy version was only administered when improving effects were not found. In addition, we examined whether PPT and DPN produced dendritic spine changes that were consistent with drug effects on learning within 40min.
Materials and Methods

Subjects

Subjects were 558 female CD1 mice (*Mus musculus*), purchased ovariectomized at 2mth of age (Charles River, QC), and tested 10-15d later. An outbred mouse strain was used so results would be more generalizable to other mouse strains. Eighteen stimulus mice were randomly chosen for social recognition paradigms, and 49 animals were used for dendritic spine analyses. Remaining mice were tested in behavioural paradigms. Mice were pair housed, then individually housed for three days. Subjects were housed with corncob bedding and environmental enrichment in clear polyethylene cages (16cm x 12cm x 26cm), on a 12:12h reversed light/dark cycle (lights off at 08:00), received rodent chow (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, WI) and water *ad libitum*. Ambient temperature was 21±1°C. Research was conducted in accordance with the Canadian Council on Animal Care and approved by University of Guelph’s Animal Care and Use Committee.

Animals were moved into the experimental room the night before testing, weighed, and vaginal smears taken and stained with Giemsa (Sigma-Aldrich, ON) to ensure completeness of ovariectomy. All behavioural tests were conducted in home cage during the dark phase of the light cycle under red light illumination.

Drugs

Mice were subcutaneously injected with 10ml/kg of the selective ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT; Stauffer, *et al* 2000; Sigma-Aldrich, ON), or ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; Meyers, *et
PPT’s vehicle was sesame oil. DPN’s vehicle was sesame oil with 2% ethanol. PPT and DPN experiments each included 5 treatment groups: vehicle, 1mg/kg, 1.67mg/kg, 2.5mg/kg, 5mg/kg, corresponding to 30µg, 50µg, 75µg, and 150µg per average 30g mouse, respectively. Injection site was sealed using Nexcare liquid bandage (3M Canada, ON) to prevent leakage. Drug treatments were assigned using a random number generator.

PPT and DPN most likely produce rapid effects through intracellular and/or membrane bound ERα and ERβ. However cannot exclude the possibility that they may activate other putative, not fully characterised membrane ERs such as ER-X (Toran-Allerand, et al 2002).

**Rapid Learning Paradigms**

Animals were injected with PPT, DPN or their vehicles 15min prior to learning paradigms. Testing was completed 40min after injection to determine rapid effects of ER agonists on learning (paradigm-specific details below). Two versions of each learning paradigm were developed. The “difficult” paradigm to assess learning enhancements was designed such that control animals would not learn because of limited learning opportunity (i.e., fewer exposures to the stimuli). The “easy” paradigm has greater learning opportunity (i.e., more stimuli exposures) and was designed such that control animals would learn. Difficult paradigms had two habituations followed by test (all 5min in duration), with 5min inter-test intervals (Figure 7). Easy paradigms had three habituations followed by test (all 4min in duration), 3min inter-test intervals (Figure 7). Even though total habituation time in the easy paradigm is only 2min longer than in the difficult paradigm, the greater number of habituations elicits enhanced stimulus
investigation, thus facilitating learning. Treatment effects were first tested using the difficult paradigm. Whenever improving effects were not observed, the easy paradigm was used with different mice to assess learning impairments. All experiments consisted of a unique set of mice; no mice were tested on more than one learning paradigm. Habituation and tests were video recorded under infrared light (8mm Handycam Nightshot, Sony, ON). Between exposures objects (Stainless steel drain catchers, glass cubes, plastic hairclips) and cylinders (described below) were washed using odourless detergent and baking soda to remove odour cues such that novelty of the stimuli remained consistent. Objects were held in position using Velcro and removed during inter-test intervals. Pilot studies indicated mice did not prefer one type of object to any other.

Social Recognition Learning Paradigm

This paradigm was modified from Choleris et al. (2006). Stimulus mice (2-4mth old, ovariectomized CD1 mice) were presented to a test mouse in clear Plexiglas cylinders with holes at the bottom, allowing passage of olfactory cues, eliciting high levels of investigation (Choleris, et al 2003; Choleris, et al 2006; Kudryavtseva, et al 2002). During habituations, the test mouse was exposed to the same two stimulus mice in consistent positions within home cage. During test, one of the two stimulus mice was replaced with a novel individual (the mouse that was replaced was counterbalanced). Stimulus mice were replaced with empty Plexiglas cylinders during inter-test intervals.

Object Recognition Learning Paradigm

During the difficult paradigm habituations, two different objects were used, while two identical objects were used during habituations in the easy version. (Pilot studies indicated this was necessary to make the paradigm ‘easy’.) One of the objects was
replaced by a novel object during test (the object replaced was counterbalanced). Objects were held in consistent positions throughout the paradigm.

**Object Placement Learning Paradigm**

A test mouse was presented with two identical objects in consistent positions (position A and position B) during habituations. During test, one of the two objects was moved to a novel location (position C). The object moved was counterbalanced.

**Olfaction Test**

Mice were weighed, moved into the experimental room, and food deprived the evening before testing (12-14hrs) to increase motivation to feed. Mice were administered sesame oil (vehicle; n=11), 50µg (n=11), or 75µg (n=9) of PPT. These doses were tested to determine whether learning effects could be explained by changes in olfaction. Mice were given ¼ chocolate chip (Hershey’s, approximately 70-80mg) 15min after injection to familiarize them with the food item. Forty minutes after injection, the mouse was distracted and ¼ of a chocolate chip was buried in a random location in the bedding of their home cage. Latency to find the chocolate chip was recorded.

**Behavioural Data Analysis**

Numbers of mice tested in each treatment group are detailed in investigation duration figure legends. Ten behaviours (listed in Table 2) were recorded for the learning paradigms using The Observer Video Analysis software (Noldus Information Technology, Wageningen, Netherlands) by four trained observers, blind to drug treatment.
Active sniffing of stimuli (nose twitching and within ~1-2mm of stimulus), was considered indication of investigation (Choleris, et al 2003; Dere, et al 2007). Since mice naturally investigate novel or displaced stimuli more than familiar ones (Choleris, et al 2003; Dere, et al 2007), stimulus investigation was used to determine whether mice recognized novel or displaced stimulus during test. Therefore, for each mouse an investigation ratio (IR) was calculated: IR=A/(A+B), where A is the investigation duration of the novel or displaced stimulus during test (or during habituations, of the stimulus to be replaced or moved at test), and B is the investigation duration of the other stimulus. Significant increases of investigation ratio from the average investigation ratio over all habituations (IR_{Hab}) to test (IR_{Test}) demonstrated novel stimulus recognition. Mice spending less than 5% of test duration investigating social stimuli (<15sec in the difficult paradigm, <12sec in the easy paradigm) or less than 3% of test duration investigating object stimuli (<9sec for the difficult paradigm, <7sec for the easy paradigm) were excluded from analysis (~5% of total animals excluded). Individuals that spent <5sec investigating each of the two stimuli during habituations (~1% of animals) and IR_{Test} outliers (>2 standard deviations ± mean; <1% of individuals) were also excluded.

**Dendritic Spine Analysis**

Golgi-Cox staining followed methods described by Gibb and Kolb (Gibb and Kolb 1998). Mice were injected with PPT, DPN or vehicle (numbers per groups are in figure captions), and returned to home cage. Forty minutes after drug injection, as per institutional Animal Care Committee guidelines, animals were sedated with CO₂ then decapitated. To limit potential effects of CO₂ on brain cytoarchitecture, the asphyxiation
was performed very rapidly (less than 1 min on average) and similarly for all groups. Brains were quickly extracted, placed into Golgi-Cox solution (1% potassium dichromate, 0.8% potassium monochromate, 1% mercuric chloride), then stored for 3-4 wk in the dark. Brains were placed in 20% sucrose (48 h at 4°C), sectioned (200 µm) using a vibrating microtome (Leica VT1000s, Leica Microsystems, ON), then stored in 6% sucrose (24 h at 4°C). Free-floating sections were placed in 4% paraformaldehyde (15 min), 1% NH₄OH (15 min), 1% Kodak rapid fixative (15-60 min), then mounted on gelatin-coated slides. Slides were air-dried at room temperature (1.5-2 h), dehydrated (50%, 70%, 95%, 100% ethanol twice, xylene twice, each for 1 min), and coverslipped with Entellan.

Images of CA1 hippocampal neurons magnified with a 63x oil objective (Figure 8; Axio Imager D1 microscope, captured with AxioCam MRc5 digital camera and AxioVision 4.6 software, Carl Zeiss, ON) and analyzed using Image J software (version 1.38x, National Institute of Health, MD) by an observer blind to the treatments. Completely stained CA1 pyramidal neurons with intact whole apical dendrites were chosen for analysis. Dendritic spine density and length were measured on apical and distal dendrites in the stratum radiatum and lacunosum-moleculare. Five CA1 neurons were analyzed per animal. Samples from secondary dendrites (>10 µm) between 40-60% (stratum radiatum) and from 80-100% (lacunosum-moleculare) the length of the apical dendrite were analyzed. Spine density was calculated as number of spines per 10 µm of dendrite length per dendritic subregion for each neuron. Spine lengths were measured from the distal tip of the spine head to the edge of the dendrite and calculated as average per dendritic subregion for each neuron.
Statistical Analysis

Behavioural data were analyzed with a two-way repeated measures ANOVAs with habituation and test as the repeated measures factor and dose treatment as a between groups factor. Specific a priori binary mean comparisons were planned to reduce the risk of type I errors. Paired t-tests within each group assessed whether preference for the novel stimulus changed from habituation (IR_{Hab} expected to be at 0.5) to test (IR_{Test} expected to be greater than 0.5 if learning has occurred). One-way ANOVAs for each dose treatment compared the preference for the novel stimulus at test (IR_{Test}) to that of the control group. For statistical analysis of investigation ratios, data were arcsin-transformed (figures represent original ratio data). The two-way repeated measures ANOVA and planned comparisons were performed for all experiments. For brevity, only significant values are reported. Latency data for olfactory test was analyzed with one-way ANOVA. One-way ANOVAs and Student-Newman-Keuls post hoc tests were used to analyze differences in dendritic spine density and average spine length, setting statistical significance at $p<0.05$. For all analyses SigmaStat version 3.5, (Systat Software, Inc., IL) was used.

Results

ERα agonist PPT

PPT improved social recognition learning at 50µg and 75µg, and slightly improved object recognition and placement learning at the 75µg dose, within 40min of injection (Figure 9). Because PPT improved learning on all 3 tasks, easy versions of these learning paradigms (used to detect learning impairments) were not tested. PPT treatment
did not enhance olfaction in the chocolate chip test (Supplemental Figure S1). PPT also significantly increased dendritic spine density in the CA1 hippocampal lacunosum-moleculare (75µg PPT) and stratum radiatum (50µg and 75µg PPT; Figure 10A,B).

There were significant main effects of PPT treatment ($F_{4,59} = 3.12, p<0.05$) and test number ($F_{1,59} = 48.48, p<0.001$) on social recognition IRs as well as a significant interaction of the main factors ($F_{4,59} = 4.56, p<0.01$). For the social recognition paradigm, planned comparisons indicated a significant main effect of PPT treatment on IR$_{Test}$ ($F_{4,59} = 4.17, p<0.01$). Post hoc analysis revealed IR$_{Test}$ values for 50µg and 75µg PPT were significantly higher than vehicle (50µg: $q=4.17$, df=25, $p<0.05$, 75µg: $q=3.45$, df=28, $p<0.05$). In addition IR$_{Test}$ was significantly higher than IR$_{Hab}$ for groups treated with 50µg ($t=4.70$, df=8, $p<0.01$) and 75µg of PPT ($t=7.09$, df=13, $p<0.001$). No other groups, including vehicle controls, demonstrated a significant difference between IR$_{Hab}$ and IR$_{Test}$. All other behaviours analyzed, including total investigation times (Figure 9B), revealed no significant effects of treatment.

Planned comparisons in the object recognition and object placement paradigms indicated the 75µg PPT group exhibited object and place recognition, since IR$_{Test}$ was significantly increased from IR$_{Hab}$ (object recognition: $t=2.70$, df=9, $p<0.05$, Figure 9C; object placement: $t=2.82$, df=11, $p<0.05$, Figure 9E). No other experimental group, including vehicle controls, demonstrated object recognition. PPT did not affect total investigation times (Figures 9D and 9F) or other behaviours recorded.

For all PPT learning paradigms, there was a significant main effect of test number for total investigation durations (all $p<0.001$). Post hoc analyses revealed significant differences between habituation 1 and habituation 2 (all $p<0.001$), as well as habituation
1 and test (all p<0.001), indicating that animals habituated to stimuli (Figure 9B,D,E), as normally observed in these paradigms (Choleris, et al 2009).

Administration of 50µg or 75µg PPT did not affect their latencies to find a buried chocolate chip in the olfaction test (Supplemental Figure S1).

There was a significant main effect of PPT treatment on dendritic spine density in the lacunosum-moleculare (F_{4,115}=3.22, p<0.05) and stratum radiatum (F_{4,115}=3.10, df=4, p<0.05). Post hoc analysis revealed PPT at 50µg and 75µg increased stratum radiatum spine density compared to vehicle (50µg: q=4.22, df=43, p<0.05, 75µg: q=4.16, df=48, p<0.05; Figure 8C,D and 10A). Dendritic spine density in the lacunosum-moleculare increased significantly with 75µg of PPT compared to vehicle (q=4.60, df=48, p<0.05; Figure 8E,F and 10B). PPT did not affect dendritic spine length (Figure 10C,D).

\textit{ERβ agonist DPN}

DPN at 75µg slightly improved object placement learning (Figure 13) but did not improve social recognition or object recognition in the difficult versions of these tasks (Figures 11A, 12A). Therefore, we tested whether DPN impaired social and object recognition using the easy versions of these paradigms. We found that DPN slightly impaired social recognition at higher doses (75µg and 150µg; Figure 11C), but did not impair object recognition (Figure 12C). DPN did not affect spines in the CA1 stratum radiatum, but decreased spine density (50µg and 150µg) and increased spine lengths at 30µg in the lacunosum-moleculare (Figure 14).

No treatment groups demonstrated social recognition in the difficult social recognition paradigm, since IR_{Test} and IR_{Hab} were not significantly different (Figure
11A). In the easy social recognition paradigm, planned comparisons revealed vehicle, 30µg, and 50µg DPN groups exhibited social recognition learning as IR_{Test} was significantly higher than IR_{Hab} (vehicle: t=2.65, df=9, p<0.05, 30µg: t=4.10, df=8, p<0.01, 50µg: t=2.94, df=11, p<0.05; Figure 11C). However 75µg and 150µg did not demonstrate learning on this task. There was no effect of DPN treatment on any other behaviour recorded, including total investigation times, during either of these experiments (Figure 11B and 11D). Hence, while DPN did not improve social recognition, it slightly impaired it at higher doses.

No groups displayed object recognition learning in the difficult object recognition paradigm (Figure 12A). All groups exhibited learning in the easy object recognition paradigm, showing significantly higher IR_{Test} than IR_{Hab} (vehicle: t=2.50, df=11, p<0.05, 30µg: t=4.44, df=11, p<0.001, 50µg: t=3.43, df=11, p<0.01, 75µg: t=3.73, df=10, p<0.01, 150µg: t=4.42, df=11, p=0.001; Figure 12C). Therefore, DPN did not rapidly modulate object recognition learning. Again, DPN did not significantly affect total investigation times (Figure 12B and 12D) or other behaviours during these two experiments.

Only animals treated with 75µg of DPN demonstrated object placement learning in the difficult object placement task, as planned comparisons revealed IR_{Test} was significantly higher than IR_{Hab} for this group (t=3.37, df=11, p<0.01; Figure 13A). Therefore, DPN at 75µg slightly facilitated object placement learning. There was no significant difference in total investigation times (Figure 13B) or other behaviours recorded, except there was a significant main effect of treatment on horizontal exploration durations (F=2.78, df=4, p<0.05). Post hoc analyses revealed that the 50µg DPN group had significantly lower horizontal exploration times than the vehicle group.
(q=4.10, df=23, p<0.05). However, these differences in horizontal exploration were not paralleled by changes on object placement performance.

For all DPN difficult and easy learning paradigms experiments, there was a significant main effect of test number for total investigation durations (all p<0.001). Post hoc analyses revealed there were significant differences between habituation 1 and habituation 2 (all p<0.001), habituation 1 and habituation 3 in the easy learning paradigms (all p<0.001), as well as habituation 1 and test (all p<0.001; Figure 11B,D, 12B,D and 13B) indicating normal habituation of the test animal to stimuli (Choleris, et al 2009). In the easy object recognition paradigm, there was also a significant increase in investigation durations at test when compared to habituation 2 and to habituation 3 (all p<0.001), indicating a dishabituation caused by the novel object during test (Choleris, et al 2009). This dishabituation was not seen in the easy social recognition paradigm, because greater habituation may be necessary to reliably see dishabituation, as in the original social recognition protocol (Choleris, et al 2009).

DPN treatment did not significantly affect spines in the stratum radiatum (Figure 14A,C). However, there were significant main effects of DPN on spine density (F_{4,120}=5.78, p<0.001) and length (F_{4,120}=3.742, p<0.01) in the lacunosum-moleculare. Post hoc analysis revealed mice treated with 50µg or 150µg of DPN had fewer spines compared to vehicle controls (50µg: q=4.49, df=48, p<0.01, 150µg: q=4.73, df=48, p<0.01; Figure 14B). Injection of 30µg of DPN increased dendritic spine length in the lacunosum-moleculare (q=4.11, df=48, p<0.05; Figure 14D).
Discussion

Rapid effects via ERα

ERα activation through PPT treatment improved social recognition at doses of 50µg or 75µg within 40min of administration (Figure 9A). Only groups administered 75µg of PPT exhibited object recognition and placement learning (Figure 9C,E). Therefore, 75µg of PPT may facilitate novel object and novel place recognition. These results appear specific to learning, as PPT treatment did not significantly affect any other behaviour recorded from these mice (e.g., horizontal exploration, rearing, total time spent sniffing stimuli), nor did it affect their olfactory capabilities (Supplemental Figure S1). It also seems unlikely that drug treatments increased interest in novelty per se, since PPT did not increase investigation times during the first habituation session when both stimuli were novel (Figure 9B,D,F).

Overall, effects of PPT appear greater for social recognition than for object recognition or placement. This may be due to greater salience of social stimuli, leading to enhanced learning about the stimuli (Choleris, et al 2009). This explanation is supported by the fact that investigation durations were approximately doubled for stimulus mice compared to object stimuli in all groups (Figure 9B, D, F). Because PPT rapidly improved performance on all three behavioural paradigms, rapid ERα activation may improve learning in general. Since we did not examine whether PPT impaired learning using the easy paradigms, we cannot exclude the possibility that higher doses of PPT may impair performance on these tasks.
Our results with PPT are consistent with previous studies reporting 17β-estradiol and 17α-estradiol enhance object recognition and placement memory consolidation when administered immediately (but not 45min or 2h) after the learning trial, when effects were assessed 4-48h after drug administration (Fernandez, et al 2008; Frick, et al 2010; Inagaki, et al 2010; Luine, et al 2003; Walf, et al 2008). To our knowledge, this is the first study including the acquisition phase in PPT’s rapid effects. The timing of our experiments (40min after drug injection and within 25min of acquisition during the transcription-independent phase of learning; Bourtchuladze, et al 1994; Da Silva, et al 2008; Nguyen, et al 1994) suggests that PPT affects early memory mechanisms. Our recent data indicate that, like PPT, 17β-estradiol improves performance on all three learning paradigms described here, suggesting that estradiol’s rapid effects during this timeframe may predominantly be mediated through ERα.

Within 40min of treatment, PPT (50µg or 75µg) increased dendritic spine density in the CA1 stratum radiatum, while 75µg PPT increased spine density in the CA1 lacunsum-moleculare (Figure 10A,B). PPT was reported to increase spine density 2h after drug application hippocampal slices from male rats (Mukai, et al 2007; Murakami, et al 2006). In cultured cortical neurons, increases in spine density occurred after 15min of incubation with 17β-estradiol (Srivastava, et al 2008), suggesting that estrogens may affect spine density within an even shorter time frame than the 40min used here. At the same time, the later effects observed in hippocampal sections (2h; Mukai, et al 2007; Murakami, et al 2006) suggest that PPT’s enhancing effects on dendritic spines may be of sufficient duration to mediate the effects on learning observed in the present study.
Increases in spine density may reflect an increase in the number of synaptic connections (Ogiue-Ikeda, et al 2008), and therefore provide new sites at which learning can occur.

To the best of our knowledge, these data provide the most rapid in vivo evidence for a PPT effect on dendritic spines and a PPT dose response relationship. Interestingly, both learning and dendritic spine experiments demonstrated PPT effects at middle doses of 50µg and 75µg, but not at lower or higher doses forming an inverted U-shaped dose response curve, which has also been reported for PPT’s longer term responses (e.g., Clipperton, et al 2008). Thus, both in terms of drug dose and timing, ERα-mediated rapid learning improvements parallel those of ERα-mediated increases in dendritic spine density.

Rapid effects via ERβ

Unlike ERα, rapid ERβ activation had mixed effects on learning. ERβ agonist DPN did not facilitate social recognition learning at any dose (Figure 11A). However, when tested using the easy social recognition paradigm, animals treated with vehicle, 30µg or 50µg of DPN exhibited social recognition learning, while those administered 75µg or 150µg of DPN did not (Figure 11C). This may indicate that DPN at higher doses impairs social recognition. Conversely, treatment with DPN neither improved nor impaired object recognition learning (Figure 12A,C), while it facilitated object placement at a dose of 75µg (Figure 13A). All treatment groups, including vehicle controls, failed to demonstrate object placement learning, with the exception of those animals treated with 75µg of DPN (Figure 13A). Thus, the middle dose of DPN enhanced object placement learning, indicating a possible inverted U-shaped dose response curve similar to that
reported for the long-term effects of another ERβ agonist (Clipperton, et al 2008). As with PPT, it seems unlikely that DPN’s effects are due to changes in interest in novelty per se, since investigation durations were unaffected during the first habituation, when all stimuli were novel (Figure 13B). Also, they do not seem to be secondary to changes in other behaviours, since DPN treatment did not significantly affect other recorded behaviours. This effect of DPN is unlikely due to changes in olfaction since pre-acquisition DPN and WAY 200070 did not impair olfaction/performance on olfactory-based tasks (Clipperton, et al 2008). Therefore, the small improvement of DPN at 75µg in the object placement paradigm suggests a specific role for ERβ in spatial learning, while higher doses of DPN may impair social recognition.

DPN did not rapidly affect dendritic spines in the CA1 stratum radiatum, but it decreased spine density in the CA1 lacunosum-moleculare at middle and higher doses (50µg and 150µg; Figure 14B), and increased lacunosum-moleculare spine length at the lowest drug dose (30µg; Figure 14D). There does not appear to be any parallel between this increased spine length and performance on learning paradigms, since groups of mice treated with 30µg of DPN did not demonstrate learning effects. Similarly, the decrease in dendritic spine density caused by 50µg and 150µg of DPN does not seem to coincide generally with any learning effects. It seems as though DPN’s effects on spine density and length may be more complicated than those of PPT. The ERβ agonist WAY-200070 rapidly increased spines in cultured cortical neurons (Srivastava, et al 2010), whereas DPN did not rapidly affect male rat CA1 hippocampal dendritic spines (Mukai, et al 2007; Murakami, et al 2006). Thus, the roles of ERα and ERβ in rapid spinogenesis may differ depending on the brain region, the species, or sex of the subjects. Clearly, literature
on rapid ERβ-mediated dendritic spine changes and their consequences for learning is incomplete and further investigations are needed.

*Rapid versus long-term effects*

Rapid effects of PPT appear to be very different than those of DPN. Whereas PPT slightly improved performance on all three learning paradigms, DPN’s learning effects were weaker and depended on the type of learning paradigm used. PPT also increased hippocampal spine density, whereas DPN either had no effect or decreased spine density. These differences resulting from selective ERα and ERβ activation are not surprising, since differences in behavioural effects are commonly seen for PPT and DPN’s long-term effects (reviewed in Choleris, *et al* 2008; Choleris, *et al* 2009). Studies with knock out (KO) mice showed ERαKO mice were completely impaired in social recognition whereas ERβKO mice, while impaired compared to wild type controls (Choleris, *et al* 2003; Choleris, *et al* 2006), could still distinguish a novel from a familiar conspecific (Choleris, *et al* 2006). These KO studies and present results suggest a greater role for ERα than ERβ in social recognition. In general, however, long-term experiments implicate ERβ in learning and memory improvements, while ERα activation generally has no effect or impairs learning and memory (Choleris, *et al* 2008; Hammond, *et al* 2009; Liu, *et al* 2008). Thus the respective role of ERα and ERβ in rapid versus longer-term effects on learning and memory may be different.

Previous work on rapid effects of 17β-estradiol or ER agonists on memory consolidation appears consistent with long-term effects of estrogens. Post-acquisition 17β-estradiol and DPN administration to wild-type, but not ERβ KO mice enhanced both
object recognition and placement memory consolidation when tested 4h or 48h after drug administration (Fernandez, et al 2008; Frick, et al 2010; Walf, et al 2008). Conversely, post-acquisition PPT did not improve memory consolidation in animals tested 48h after administration (Frick, et al 2010). Our study is the first to include learning acquisition when examining the rapid effects of ER agonists, and to test animals during a time when memory is transcription-independent (Bourtchuladze, et al 1994; Da Silva, et al 2008; Nguyen, et al 1994). Our results suggest the role of ERα and ERβ rapid effects may be different during different stages of memory processing.

on hippocampal spinogenesis, both ERα and ERβ may be involved in estradiol’s longer-term effects.

**Conclusions**

To the best of our knowledge, this is the first report demonstrating *in vivo* effects of estrogen receptor agonists on CA1 dendritic spines and learning only 40min after drug injection. This time frame is consistent with the rapid effects of estrogens on neuronal electrophysiology and cell signalling mechanisms. Our results suggest both ERα and ERβ activation can rapidly influence learning and synaptic connections, but that effects are dependent on the estrogen receptor and the type of learning studied. At the time point studied, ERα seems to have a greater role in promoting estrogen-mediated enhancements in learning and memory processes and CA1 hippocampal dendritic spine increases than ERβ.

Studies on rapid learning effects of estradiol and ER agonists may help to explain the often inconsistent literature on estradiol’s learning and memory effects (reviewed in Choleris, *et al* 2008), since physiologically, estradiol would exert rapid and genomic effects at the same time (Vasudevan and Pfaff 2008). It has been hypothesized that differential activation of ERα and ERβ, their involvement in different types of learning, different stages in memory processing, could all contribute to estrogen effects on learning and memory (Choleris, *et al* 2008). Our study suggests that timing of estrogen exposure (short versus long-term) and the specific cellular mechanisms involved (non-genomic, genomic, and their interactions; Vasudevan and Pfaff 2008) may also be important determinants of learning effects.
Acknowledgements

We thank Skot koshowski, Alexandra Muller, Dana Munroe, and Dima Saab for their help with data collection.
Table 2: Description of behaviours collected and analyzed from learning experiments.

<table>
<thead>
<tr>
<th><strong>Behaviour</strong></th>
<th><strong>Description</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniff Stimulus</td>
<td>Sniff/Investigation of object or mouse stimulus</td>
</tr>
<tr>
<td>Bite Stimulus</td>
<td>Biting object or mouse stimulus</td>
</tr>
<tr>
<td>Sit/Climb on Stimulus</td>
<td>All 4 paws off the cage floor and sitting/climbing on object stimulus</td>
</tr>
<tr>
<td>Dig</td>
<td>Movement of forepaws to propel bedding posteriorly</td>
</tr>
<tr>
<td>Bury</td>
<td>Movement of forepaws to push bedding away from the body</td>
</tr>
<tr>
<td>Horizontal Exploration</td>
<td>Walking around the cage, including non-stimulus sniffing</td>
</tr>
<tr>
<td>Rearing</td>
<td>Both forepaws off the ground</td>
</tr>
<tr>
<td>Self Groom</td>
<td>Movement of forepaws over face and body</td>
</tr>
<tr>
<td>Inactivity</td>
<td>Includes sit, lay and sleep</td>
</tr>
<tr>
<td>Stereotypies</td>
<td>Strange, repetitive behaviours (greater than 3 repetitions), including jumps, head shakes, lid chews</td>
</tr>
</tbody>
</table>
Figure 7: A comparison of the time line of events for the difficult and easy behavioural paradigms (H indicates habituations). Both learning paradigms begin 15min after drug injection, and are completed within 40min of injection.
Figure 8: Images of hippocampus or CA1 hippocampal neurons. Pyr indicates pyramidal cell layer, rad indicates the stratum radiatum and l-m the lacunosum-moleculare. (A) Nissl stained hippocampus section, scale bar 400µm. (B) Camera lucida drawing of Golgi stained hippocampal primary neuron, scale bar 100µm. (C-J) Microscope images of Golgi stained CA1 secondary dendrites in the stratum radiatum and lacunosum-moleculare from animals treated with PPT vehicle, 75µg of PPT, DPN vehicle, or 150µg DPN. Scalebars indicate 5µm.
Figure 9: Rapid effects of PPT on learning paradigms. Asterisks above black bars in A, C, and E represent a significant difference between the investigation ratio (IR) at
habituation versus the IR at test for the treatment. (A) PPT at doses of 50µg and 75µg significantly improved social recognition above vehicle treated animals (indicated by an asterisk above lines over the 50µg and 75µg PPT and vehicle controls). (B) Total investigation times during the social recognition experiment were not affected by PPT treatment. (C) The group receiving 75µg of PPT was able to successfully perform the object recognition task. (D) Total investigation durations for the object recognition experiment did not differ with PPT treatment. (E) Mice administered 75µg of PPT were successfully able to perform the object placement task. (F) PPT treatment did not significantly affect total investigation durations for the object placement experiment. Means and standard error are depicted. *p<0.05, **p<0.01, ***p<0.001.
Figure 10: Rapid effects of PPT on dendritic spine density and length in the CA1 hippocampus. All groups contain measures of 25 neurons from 5 animals, except the 50µg PPT group which contain measures of 20 neurons from 4 animals. (A) PPT at 50µg and 75µg increased spine density in the stratum radiatum. (B) PPT at a dose of 75µg increased spine density in the lacunosum-moleculare. (C) PPT does not affect spine lengths in the stratum radiatum. (D) Spine lengths in the lacunosum-moleculare are also not affected by PPT treatment. Means and standard error are depicted. *p<0.05
Figure 11: Rapid effects of DPN on social recognition. Asterisks above black bars in A and C represent a significant difference between the investigation ratio (IR) at habituation versus the IR at test for the treatment. (A) DPN treatment had no improving effects on social recognition as assessed by the difficult social recognition task. (B) Treatment with DPN also did not affect total investigation durations for the difficult social recognition experiment. (C) Animals treated with vehicle, 30µg, or 50µg of DPN were able to successfully recognize the novel conspecific at test in the easy version of the social recognition paradigm. (D) Investigation durations during the easy social recognition experiment were not affected by DPN treatment. Means and standard error are depicted. *p<0.05, **p<0.01
Figure 12: Rapid effects of DPN on object recognition. Asterisks above black bars in A and C represent a significant difference between the investigation ratio (IR) at habituation versus IR at test for the treatment group. (A) DPN treatment did not rapidly improve object recognition in the difficult version of this paradigm. (B) DPN treatment also did not affect the total investigation durations during the difficult object recognition experiment. (C) In the easy object recognition experiment, all groups (vehicle, 30µg, 50µg, 75µg and 150µg of DPN) were able to successfully perform the task. (D). Investigation durations for the easy object recognition experiment were not affected by DPN treatment. Means and standard error are depicted. *p<0.05, **p<0.01, ***p<0.001
Figure 13: Rapid effects of DPN on object placement. Asterisks above black bars in A represent a significant difference between the investigation ratio (IR) at habituation versus IR at test for the treatment group. (A) The group administered 75\( \mu \)g of DPN was able to successfully distinguish the novel object placement in the difficult version of the object placement task. (B) DPN treatment did not affect total investigation durations during the object placement experiment. Means and standard error are depicted. *\( p<0.05 \)
Figure 14: Rapid effects of DPN on dendritic spine density and length in the CA1 hippocampus. All groups contain measures of 25 neurons from 5 animals. (A) DPN did not affect dendritic spine density in the stratum radiatum. (B) Treatment with 50µg and 150µg of DPN decreased spine density in the lacunosum-moleculare. (C) DPN treatment did not significantly affect spine lengths in the stratum radiatum. (D) Treatment with 30µg of DPN significantly increased spine lengths in the lacunosum-moleculare. Means and standard error are depicted. *p<0.05.
Supplemental Figure S1: Chocolate chip olfaction test: Latencies of mice to find a buried piece of chocolate chip. Vehicle n=11, 50µg n=11, 75µg n=9. Mean±SEM.
CHAPTER 4: The Hippocampus Mediates Estrogens’ Rapid Effects on Learning Through ERα
**Abstract**

We previously reported 17β-estradiol and estrogen receptor (ER) α agonist PPT rapidly improved 3 different types of learning and increased CA1 hippocampal dendritic spines within 40min of systemic administration, while ERβ agonist DPN only improved spatial learning and either did not affect spine density or decreased them (Phan, et al 2011; Phan, et al 2012). To determine the role of the hippocampus in these rapid learning enhancements, we delivered 17β-estradiol (25, 50, 100nM), PPT (50, 100, 150nM) and DPN (50, 100, 150nM) or vehicle directly into the dorsal hippocampus of ovariectomized female CD1 mice, 15min before training and testing on an object placement, object recognition, and social recognition paradigm. Testing was completed 40min after hormone administration. Intrahippocampal 17β-estradiol and PPT improved performance on all three learning paradigms, while DPN only improved object placement learning, replicating our systemic results. Next, we examined whether the same doses of 17β-estradiol, PPT and DPN affected AMPA and NMDA receptor mediated membrane depolarizations in CA1 pyramidal neurons using whole-cell patch-clamp recordings in pre-pubescent female CD1 mice. Current-clamp recordings were performed and (S)-AMPA and NMDA was bath applied after 15-25min (corresponding to time of learning acquisition in the behavioural experiments) of 17β-estradiol, PPT, DPN or vehicle, using the same doses as above. Interestingly, we found that 17β-estradiol- and PPT-mediated rapid learning enhancements corresponded with an attenuation in AMPA receptor, but not NMDA receptor, mediated membrane depolarizations. DPN had no effect. Thus the
hippocampus is able to mediate estrogens’ rapid effects on learning. These effects may be mediated by the creation of immature synapses.

**Introduction**

Estrogens have been shown to affect the central nervous system to influence a variety of behaviours via two general pathways, through non-genomic (or rapid; <1-2hrs) and genomic actions (24-48hrs), of which the latter is better characterized (reviewed in Choleris, *et al* 2008). Recently, we have shown that systemic injections of 17β-estradiol to female ovariectomized mice rapidly improved learning on a spatial task, object discrimination and social discrimination when administered 15min before learning acquisition and tested within 40min of administration (Phan, *et al* 2012). These systemic effects appear to be mediated predominantly through ERα, rather than through ERβ. Administration of an ERα agonist improved performance on all 3 learning paradigms, similar to the effects of 17β-estradiol (Phan, *et al* 2011). Administration of an ERβ agonist, however, improved spatial learning only, with no effects on object discrimination and possibly impairing effects on social discrimination (Phan, *et al* 2011). Furthermore, others have previously found that spatial and object discrimination memory consolidation in female rats and mice was improved when estrogens were systemically administered immediately after learning, and memory retention was tested 4-48hrs later (Fan, *et al* 2010; Fernandez, *et al* 2008; Gresack and Frick 2006; Inagaki, *et al* 2010; Luine, *et al* 2003; Packard and Teather 1997b; Walf, *et al* 2006; Walf, *et al* 2008).

Estrogens’ rapid effects on learning and memory are likely due to increased neural plasticity in brain areas that are important to the specific tasks tested. For example,
the hippocampus appears to be able to mediate some of estrogens’ rapid enhancing
effects on spatial and object memory consolidation, since intrahippocampally
administered estrogens into rats and mice immediately after learning acquisition
improved their performance on these memory tasks when tested 24-48hrs later (Fan, et al
2010; Fernandez, et al 2008; Packard and Teather 1997a). In support of a role for the
hippocampus in estrogen-mediated learning and memory enhancements, numerous
studies have found that estrogens rapidly affect the plasticity of hippocampal neurons,
especially within the CA1 region. Estrogens rapidly increased dendritic spine or synapse
density within the hippocampus of rats (MacLusky, et al 2005; Mukai, et al 2007;
Murakami, et al 2006). In addition, we have also recently shown that 17β-estradiol and
an ERα agonist increased dendritic spine density within the CA1 hippocampus of mice,
in a manner that was consistent with the timing and doses of estrogens needed to rapidly
however had no effect or decreased dendritic spine density in the CA1 pyramidal neurons
(Phan, et al 2011). In addition to these changes in synapse density, estrogens increased
hippocampal excitability in rats (Foy, et al 1999; Foy and Teyler 1983; Gu and Moss
Smejkalova and Woolley 2010; Teyler, et al 1980; Wong and Moss 1991; Wong and
Moss 1992; Wong and Moss 1994) and in mice (Foster 2012), although the mechanisms
through which these were reported to occur are varied (e.g., through increasing/enhancing
NMDA, AMPA or kainate receptors, presynaptic glutamate vesicular release and/or
through reducing inhibitory neurotransmission). Estrogens’ rapid enhancement of
hippocampal excitability may contribute to estrogens’ rapid facilitation of learning and
memory. However, due to the different methodologies inherent to these studies (e.g., dose ranges: pM-nM in electrophysiology experiments, µg/kg-mg/kg in behavioural experiments; timing: ~10min-1hr of drug application in electrophysiology experiments, 4-48hrs after post-learning hormone administration in the majority of behavioural experiments; see above for references), comparison between electrophysiological and behavioural experiments is difficult. Therefore, to the best of our knowledge, it is not known whether these estrogen-induced changes in hippocampal electrophysiology occur at similar doses and timing with respect to their rapid behavioural learning effects.

These studies clearly demonstrate that estrogens non-genomically affect the plasticity of hippocampal neurons, and suggest the hippocampus may mediate at least some of estrogens rapid effects on learning. To determine the extent to which the hippocampus is able to mediate estrogens’ rapid facilitation of object placement, object recognition and social recognition demonstrated in systemic experiments (Phan, et al 2011; Phan, et al 2012), we replicated these learning paradigms using intrahippocampal hormone delivery. In addition, we also examined whether ERα or ERβ were responsible for this effect through the use of receptor selective agonists. Drugs were administered intrahippocampally 15min prior to learning acquisition, and the tasks were completed within 40min of hormone manipulation. This procedure allowed us to test the non-genomic effects of estrogens, since memories are maintained by second messenger systems during this time, and are independent of protein synthesis (reviewed in Kandel 2001). We then examined the effects of 17β-estradiol, and an ERα and ERβ agonist on CA1 hippocampal AMPA and NMDA receptor mediated neurotransmission within 15-30min of drug application (glutamate receptors important for learning and memory, Bliss
and Lomo 1973; Martin, et al 2000). This 15-30min time frame corresponds to the duration when learning acquisition occurs in the behavioural paradigms, in an effort to elucidate how estrogens may rapidly affect hippocampal electrophysiology to improve learning (Phan, et al 2011; Phan, et al 2012).

**Materials and Methods**

**Subjects**

Female CD1 mice (*Mus musculus*) were ovariectomized, implanted with bilateral cannulae and used for learning experiments (2.5 months old; Charles River, Saint-Constant, QC, Canada). Pregnant female CD1 mice were purchased (14-16 days gestation; Charles River, QC), and female pups 20-30 days old were used for electrophysiology experiments.

All animals were housed in the Central Animal Facility, University of Guelph, on a reversed light/dark cycle (12:12h, lights on at 2000h) at 21±1°C. Mice were held in clear polyethylene cages (26cm x16cm x 12cm) with corncob bedding, environmental enrichment, and were provided with tap water and rodent chow *ad libitum* (14% Protein Rodent Maintenance Diet or 18% Rodent Diet, Harlan Teklad, WI). Mice tested in learning paradigms were individually housed. Cages were not changed for at least 3 days prior to experiments (for establishment of a home cage territory), while stimulus mice were group housed. Litters of pups for electrophysiology experiments were group housed and remained with their mother until used in electrophysiology experiments.

The evening before behavioural testing, mice were moved into the testing room to acclimate. All test mice were only used in one learning experiment. Conducted in
accordance with the Canadian Council on Animal Care, approved by University of
Guelph’s Animal Care and Use Committee.

**Learning Experiments**

**Surgery: Ovariectomy and Cannulation**

Adult mice were ovariectomized as previously described in (Clipperton Allen, *et al* 2011). Briefly, mice were anaesthetized (isoflurane), placed in a stereotaxic frame
using atraumatic ear bars (David Kopf instruments, CA), subcutaneously (s.c.) injected
with an analgesic and anti-inflammatory (carprofen 50mg/kg; Rimadyl, Pfizer Canada
Inc, Kirkland, QC, Canada). Ovaries were removed from the dorsal side, drops of local
anaesthetic were dripped onto the incision (mix of bupivacaine 0.17% (Hospira, Inc.,
Montreal, QC, Canada) and lidocaine 0.67% (Alveda Pharmaceuticals, Toronto, ON,
Canada), and closed with a surgical clip (MikRon Autoclip 9mm wound clips, MikRon
Precision Inc., Gardena, CA). Immediately following the ovariectomy, the skin on the
dorsal surface of the mouse skull was excised, local anaesthetic was dripped onto the
incision site, as above. Two holes were drilled for 26 gauge bilateral guide cannulae
aimed at the anterior dorsal hippocampus (Plastics One, HRS Scientific, Anjou, QC,
Canada), at stereotaxic coordinates 1.7mm posterior to bregma and 1.5mm lateral to
midline, 1.3mm below skull surface (Paxinos and Franklin 2001). Injectors extended
1mm beyond the end of the guide cannula, for a final dorsal-ventral position of 2.3mm
below the skull surface. Three holes were drilled in a triangular pattern around the guide
cannulas into which 3 jewellers screws were screwed. Dental cement (Central Dental Ltd,
Scarborough, ON, Canada) was used to form a headcap around the screws and guide
cannulae. Dummy cannulae (flush with guide cannulae) were inserted. Mice received a rehydrating intraperitoneal injection of 0.5mL of saline postsurgery. Experiments were performed 10-15 days after surgery.

**Rapid Learning Paradigms**

Each experiment consisted of a unique set of experimentally naïve mice. All drugs were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Animals were gently restrained by hand and microinfused with 17β-estradiol (25nM, 50nM or 100nM), PPT (50nM, 100nM or 150nM), DPN (50nM, 100nM or 150nM) or vehicle (0.01-0.02% ethanol in artificial cerebral spinal fluid; aCSF, in mM; 126 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 D-glucose, 1.25 NaH₂PO₄), 0.5µL per hemisphere at a rate of 0.2µL per minute using a microinfusion pump (PHD 2000, Harvard Apparatus, QC, Canada). Mice received drug infusions 15min prior to testing. Each learning paradigm was completed within 40min of drug administration, thus targeting the *rapid* effects of 17β-estradiol, PPT and DPN. Learning paradigms consisted of 2 habituation sessions and a test, each 5min in duration, separated by 5min intervals (previously described in Phan, *et al* 2011). The learning paradigms were designed so that vehicle controls do not demonstrate learning, in order to test for improving effects of treatments. When given greater numbers of habituations, vehicle-treated control mice can perform these tasks (see Phan, *et al* 2011).

All behavioural paradigms were conducted in home cage under red light, during the dark phase of the light cycle. Habituation and test sessions were recorded under infrared light (8mm Handycam Nightshot, Sony, Cambridge, ON, Canada) for
ethological analysis. During all intertest intervals, objects and cylinders used to present stimulus mice (described below) were washed to remove odour cues using an odourless detergent and baking soda. Objects were held in place using Velcro, and were previously tested to ensure mice did not have an initial preference for any one object. Objects consisted of a glass cube, stainless steel drain catcher, and plastic hairclip.

After behavioural testing, mice were intrahippocampally infused with Chicago blue dye (1% in phosphate buffered saline (PBS)) to check cannula placements (supplemental figures S2, S3, and S4). Vaginal smears were taken and stained with Giemsa. Cell morphology was examined to ensure effectiveness of ovariectomy, as described in Oksjoki, et al 1999. Whole brain tissue was extracted and fixed in 4% paraformaldehyde for 2 weeks, placed in 30% sucrose PBS for 3-5 days, frozen at -80°C, sectioned coronally at 30µm thickness using a cryostat (Leica CM 1850, Leica Microsystems, Richmond Hill, ON), mounted on slides and coverslipped using DPX mountant. Site of injectors in the hippocampus for all experiments have been provided in Supplemental Figures S2, S3, and S4, and any animals with off target cannula were excluded from analysis (1 mouse).

**Social Recognition Learning Paradigm**

Eighteen 2.5-4 month old ovariectomized female mice were randomly chosen as stimulus animals for social recognition experiments. They were presented to experimental mice in clear Plexiglas cylinders with perforations at the bottom (to which stimulus animals had previously been habituated), allowing passage of olfactory cues (as described in Choleris, et al 2006). During habituations, a test mouse was presented with the same two stimulus mice (e.g., A and B) in consistent positions. During test, one of the
two stimulus mice was replaced with a novel mouse (e.g., A and C). The individual replaced was counterbalanced.

*Object Recognition Learning Paradigm*

Two different objects were presented to the test mouse in consistent positions during habituations, while one of the two objects was replaced with a novel third object during test. The object that was replaced was counterbalanced, and the two positions of the objects remained consistent throughout the paradigm.

*Object Placement Learning Paradigm*

Two identical objects were placed in 2 consistent locations against one side of the cage during habituations. During test, one of the objects was moved to a novel location on the opposite side of the cage. The object moved was counterbalanced.

*Behavioural Data Analysis*

Specific numbers of mice used for behavioural learning paradigms are detailed in figure legends for investigation durations (n=7-13 per group; Figure 15B, D and F). Ten behaviours listed in Table 3 were collected from each learning experiment using The Observer Video Analysis software (Noldus Information Technology, Wageningen, Netherlands) by 1 of 5 observers blind to drug treatment.

Active sniffing of stimuli (nose twitching within ~1-2mm of the stimulus) was considered investigation behaviour (Galef 2013). Mice naturally investigate (sniff) novel or displaced stimuli more than familiar ones. Therefore, we calculated Percent Investigation = N/(N+F) x 100, where N is the time spent investigating a novel or displaced stimulus (or during habituations, the stimulus that will be replaced/displaced)
and F is the time spent investigating the familiar stimulus. Investigation percent during habituations is roughly 50% (chance), while if the experimental mice recognize the novel or displaced stimulus during test, investigation percent is statistically greater than at habituation (Phan, et al 2011, 2012). Animals with total investigation durations of less than 5sec during test (4% of animals) as well as outliers (>2SDs ± mean; 2% of animals) were excluded.

Two-way repeated measures ANOVAs were used to analyze behavioural data, with factors habituations and test (repeated measure) and treatment (between groups). Investigation percent for statistical analysis was expressed as a ratio and were arcsin transformed. These preference scores for habituation 1 and 2 were averaged. To reduce type I errors, specific a priori binary mean comparisons were planned in the statistical model to assess changes in preference score at test (the experimental condition). Specifically, within the ANOVA model paired \( t \) tests were used to assess differences in preference scores between habituation and test within each treatment group, and one-way ANOVAs and Student-Neuman-Keuls (SNK) post hocs were used to assess differences in preference scores at test between doses. Unless meaningful, non-significant results are not reported.

**Electrophysiology Experiments**

A total of 133 neurons from 67 female mice (post natal day 20-30) were patch clamped. Animals were anesthetized with isoflurane, decapitated, and brain tissue was extracted in oxygenated (95% \( O_2 \), 5%\( CO_2 \)) ice-cold sucrose aCSF (in mM; 342 sucrose, 180 D-glucose, 84 NaHCO\(_3\), 111 CaCl\(_2\), 120 MgSO\(_4\), 75 KCl, 120 NaH\(_2\)PO\(_4\)), where they were coronally sectioned using a vibrating microtome (Leica VT1000x, Leica...
Microsystems, Richmond Hill, ON) at 200µm thickness. Sections of anterior dorsal hippocampus (coinciding with cannula placements in behavioural experiments) were placed in oxygenated aCSF (in mM; 58 NaCl, 180 D-glucose, 84 NaHCO₃, 111 CaCl₂, 120 MgSO₄, 75 KCl, 120 NaH₂PO₄) at 32°C to recover for at least 1hr before whole-cell patch-clamp recording.

Borosilicate glass recording electrodes (4-7MΩ) were pulled using a micropipette puller (P-97, Sutter Instrument Company, CA) and filled with intracellular solution (in mM; 140 K-gluconate, 5 EGTA, 2 MgCl₂, 1 CaCl₂, 0.6 NaHCO₃, 10 HEPES, 2 Mg-ATP, 2 Na₂-ATP, 0.3 Na-GTP, 8.3 sucrose, 1% biocytin). CA1 hippocampal neurons were visualized using an infrared filter (Carl Zeiss, Toronto, Canada) and patched using Multiclamp 700B and Clampex software (version 10, Molecular Devices, CA). Recordings of membrane potential (current-clamp mode) were taken from separate CA1 hippocampal neurons. Only one current-clamp recording from one neuron per brain section was taken. All solutions were at room temperature, and a flow rate of 1.5-2mL/min was used during recordings. After 10min of recording in aCSF, 0.2-0.75µM of (S)-AMPA or 10-15µM of NMDA (Tocris Bioscience, Minneapolis, MN) was bath applied for 15sec-1.5min to elicit, on average, a 6-7mV (AMPA) or 5mV (NMDA) amplitude depolarizations (baseline measure). Depolarizations were maintained at subthreshold levels. Following this baseline measure, 17β-estradiol (25nM, 50nM, or 100nM), PPT (50nM, 100nM or 150nM) DPN (50nM, 100nM, or 150nM), or their vehicle control (0.02% ethanol in aCSF) was bath applied for 15-20min. These doses of estrogen and agonists are the same doses delivered intrahippocampally for the learning experiments. Then, AMPA or NMDA was reapplied (using the same dose and timing as
for the baseline application) in the presence of 17β-estradiol, PPT, DPN or the vehicle control. After this response was recorded, regular aCSF was bath applied for washout period of 10-15min, and a third measure for AMPA or NMDA depolarization was taken (recovery measure). Neurons that sensitized or desensitized (gradually increasing or decreasing responses from baseline to recovery) to either AMPA or NMDA during the course of recordings were excluded from the data set (17 neurons) One-way ANOVAs and Student-Neuman-Keuls post hocs were used for analysis. Non-significant results are not reported, unless meaningful.

**Results**

*Magnetic Resonance Imaging (MRI) and Hemodynamics*

**Intrahippocampal Delivery of 17β-estradiol**

Intrahippocampal 17β-estradiol improved object placement, object recognition and social recognition learning within 40min of administration (Figure 15).

The object placement and object recognition experiments resulted in a significant effect of treatment on investigation percent at test assessed by a one-way ANOVA ($F_{3,38}=3.138$, $p<0.05$ and $F_{3,42}=9.559$, $p<0.001$, respectively). *Post hocs* revealed that for both experiments, the 50nM 17β-estradiol group performed significantly better in the object placement and recognition paradigms than did vehicle controls ($q=3.868$, $df=18$, $p<0.05$ and $q=7.133$, $df=22$, $p<0.001$, respectively; Figure 15A, 15C). Throughout all three experiments, the 50nM 17β-estradiol group was the only group that successfully demonstrated learning. Paired t-tests indicated a significantly higher investigation percent at test compared to habituation for the object placement ($t=-2.552$, $df=10$, $p<0.05$; Figure
110A), object recognition (t=−8.281, df=11, p<0.001; Figure 15C), and social recognition (t=3.384, df=11, p<0.01; Figure 15E) experiments. Therefore, 50nM of intrahippocampal 17β-estradiol improved object placement, object recognition and social recognition learning.

There were significant differences in the total investigation durations across the three experiments. There was a significant interaction of test and treatment for the object placement experiment ($F_{6,76}=2.645$, p<0.05). The vehicle treated animals had significantly higher total investigation durations than the 100nM group only during habituation 1 (q=3.860, df=17, p<0.05; Figure 15B). There was also a significant effect of treatment for the object recognition experiment ($F_{3,84}=3.697$, p<0.05) and the social recognition experiment ($F_{3,80}=5.034$, p<0.01). Post hoc analysis reveals that the 50nM 17β-estradiol group had significantly higher total investigation durations compared to the vehicle control group in the object recognition experiment (q=3.537, df=22, p<0.05; Figure 15D). Conversely, for the social recognition experiment, post hoc analyses indicated the vehicle had significantly higher total investigation durations compared to all 17β-estradiol treatment groups (25nM: q=5.274, df=20, p<0.01, 50nM: q=3.891, df=21, p<0.05, 100nM: q=3.528, df=18, p<0.05; Figure 15F).

There were a few significant differences between treatment groups and other behaviours recorded from the mice during the experiments. In the object placement experiment, the two-way repeated measures ANOVA for inactivity duration failed the test for equal variances, thus a Kruskal-Wallis one-way ANOVA on ranks was used to examine this measure. The 50nM 17β-estradiol treatment group had higher durations of inactivity only during habituation 2 compared to vehicle controls ($H=10.184$, df=3, p<0.05).
p<0.05, Dunn’s post hoc Q=3.124, df=19, p<0.05). For the social recognition experiment, a two-way repeated measures ANOVA indicate there was a significant main effect of treatment (F_{3,79}=6.818, p<0.001) in rearing duration. The 25nM and 50nM 17β-estradiol group had significantly higher rearing durations than vehicle controls (q=3.910, df=20, p<0.01 and q=5.035, df=21, p<0.01, respectively). There were no effects of treatment on other behaviours recorded.

**Intrahippocampal Delivery of ERα Agonist PPT**

Intrahippocampal PPT may enhance object placement, object recognition and social recognition learning. Hence, ERα activation in the hippocampus appears to improve learning (Figure 16).

Only groups treated with intrahippocampal PPT were able to successfully demonstrate learning during all three experiments, whereas the vehicle controls could not. Groups of mice administered 100nM and 150nM of PPT into the hippocampus had an investigation percent at test significantly higher than at habituation in the object placement learning paradigm (t=5.790, df=12, p<0.001, and t=3.296, df=10, p<0.01, respectively; Figure 16A). In the object recognition experiment, all groups of mice that were treated with PPT were able to discriminate the novel object at test. Investigation percent at test was significantly higher than at habituation for mice treated with 50nM (t=3.883, df=7, p<0.01), 100nM (t=4.158, df=11, p<0.01) and 150nM (t=2.900, df=9, p<0.05) PPT (Figure 16C). Administration of 100nM of PPT improved social recognition learning significantly above vehicle. There was a significant main effect of treatment on investigation percent at test (F_{3,40}=3.128, p<0.05). Post hoc analyses
revealed that the 100nM PPT treatment group had significantly higher investigation percent at test compared to vehicle (q=3.921, df=22, p<0.05; Figure 16E). Groups administered 100nM and 150nM of PPT intrahippocampally in the social recognition experiment were able to discriminate the novel individual during test (Figure 16E). Investigation percent at test was higher than at habituation for mice treated with 100nM (t=5.177, df=10, p<0.001) and 150nM (t=4.392, df=10, p<0.01) PPT intrahippocampally (Figure 16F).

There were no effects of treatment on total investigation durations for the object placement and object recognition experiments (Figure 16B, 16D). There was a significant effect of treatment on inactivity durations during the object placement experiment (F_{3,80}=3.785, p<0.05). Post hocs indicated that the vehicle control had significantly higher inactivity durations compared to all groups that received PPT treatment (50nM: q=4.028, df=18, p<0.05; 100nM: q=4.091, df=22, p<0.05; 150nM: q=3.359, df=20, p<0.05). No other behaviours were significantly affected by PPT treatment during the object placement or object recognition experiments.

*Intrahippocampal Delivery of ERβ Agonist DPN*

Application of DPN directly into the hippocampus improved object placement learning, but did not improve object recognition or social recognition performance (Figure 17).

The object placement experiment revealed a significant effect of treatment on investigation percent at test, as analyzed using a one-way ANOVA (F_{3,38}=4.008, p<0.05). *Post hoc* analysis indicates that the 100nM DPN treatment group had a higher investigation percent compared to the vehicle controls (q=3.660, df=21, p<0.05; Figure
In addition, the 100nM DPN group was the only treatment group that was able to demonstrate object placement learning, as this group had a significantly higher investigation percent at test compared to at habituation \((t=2.671, \text{df}=9, \ p<0.05)\). No other treatment groups, including the vehicle controls, were able to discriminate the displaced object at test. There was a significant main effect of treatment on total investigation \((F_{3,76}=3.531, \ p<0.05)\) and burying \((F_{3,76}=5.228, \ p<0.05)\) durations during the object placement experiment. *Post hoc* tests revealed the 150nM DPN group had significantly higher investigation \((q=4.165, \text{df}=19, \ p<0.05; \text{Figure 17B})\) and burying \((q=4.111, \text{df}=19, \ p<0.01)\) durations compared with the vehicle control group. However, these differences in behaviour did not seem to affect their performance on the object placement task.

Intrahippocampal DPN did not improve object recognition or social recognition learning (Figure 17C, 17E). One-way ANOVAs did not reveal any significant differences in investigation percent between treatment groups, nor were there any significant differences between investigation percent at habituation and test (Figure 17D, 17F). Therefore, no groups, including the vehicle controls, were able to successfully distinguish the novel object or social stimulus in the object and social recognition learning paradigms. Moreover, intrahippocampal treatment with DPN did not affect any other behaviours recorded in the mice during both the object and social recognition experiments.

*Habituation to Stimuli - Investigation Durations*

All groups across experiments showed natural habituation to the stimuli. Total investigation durations were significantly different across test number \((\text{all } F>34.932, \text{all})\).
p<0.001). Mice habituated to the stimuli, since investigation durations at habituation 1 were significantly higher compared with habituation 2 and compared with test (all q>8.387, all p<0.001).

*Patch-clamp recordings*

Application of 17β-estradiol and PPT to hippocampal neurons for 15min decreased the amplitude of AMPA mediated membrane depolarisations (Figure 18, 20). DPN had no effect on AMPA membrane depolarisations (Figure 18, 20). In contrast, NMDA mediated membrane depolarisations were unaffected by 17β-estradiol, PPT or DPN (Figure 19, 20).

A one-way ANOVA revealed a significant main effect of 17β-estradiol treatment on the percent change of AMPA membrane depolarisation amplitude (F$_{3,19}$=4.045, p<0.05). *Post hocs* indicated that the group treated with 50nM 17β-estradiol had significantly lower percent change in AMPA membrane depolarisation amplitudes compared with vehicle controls (q=4.447, df=8, p<0.05; Figure 18B, 20A). Similarly, a one-way ANOVA resulted a significant main effect of PPT treatment on the percent changes in AMPA membrane depolarisations (F$_{3,25}$=3.326, p<0.05). *Post hocs* revealed that neurons treated with 100nM and 150nM of PPT resulted in a significantly lower AMPA depolarisation percent change compared to vehicle control (q=3.911, df=14, p<0.05 and q=3.731, df=13, p<0.05, respectively; Figure 18C, 20B). DPN did not significantly affect AMPA membrane depolarisations (Figure 18D, 20C), and there was no significant effect of any treatment on NMDA membrane depolarisations (Figure 19, Figure 20D-F).
Discussion

Intrahippocampal Learning Experiments

Here, we find that microinfusion of 50nM 17β-estradiol into the hippocampus improved object placement, object recognition and social recognition learning within 40min of administration (Figure 15). Furthermore, we show that intrahippocampal administration of the ERα agonist PPT may also rapidly facilitate performance on all three learning paradigms (Figure 16), whereas intrahippocampal ERβ agonist DPN only rapidly improves object placement learning, but does not improve object or social recognition (Figure 17). The results of our current study are fully consistent with our previous findings with systemic administration of 17β-estradiol, as well as ERα and ERβ agonists (Phan, et al 2011; Phan, et al 2012). Thus, 17β-estradiol (possibly through ERα activation) appears to rapidly improve learning in general, and estradiol action within the hippocampus is capable of mediating this effect. Ongoing work in our lab has also found that systemic administration of GPER agonist G1 produces effects similar to ERα activation and 17β-estradiol, albeit these effects are weaker than those of 17β-estradiol (Gabor, et al 2012). Therefore, we suggest that the rapid effects of 17β-estradiol in the hippocampus on learning are likely mediated through a combination of ERα and GPER activation. Interestingly, ERβ activation does have rapid effects on learning, however they are limited only to the enhancement of spatial learning. The reason for this very selective effect of ERβ remains unknown. However, it seems clear that ERα and ERβ have two separate mechanisms of non-genomic action within the hippocampus.
These effects of intrahippocampal estrogens seem specific to learning rather than hormone effects on other aspects of behaviour such as novelty interest or arousal. While we did find a few significant differences between treated groups and vehicle controls for a few behavioural measures, these effects are inconsistent across the paradigms performed (often confined only to one habituation or test session), and in general do not explain the learning effects we detect (Figures 15, 16, 17). The one exception is the higher investigation duration detected in the 17β-estradiol 50nM group during the object recognition paradigm (Figure 15D). This increase in investigation duration may explain facilitation of object recognition learning and/or be interpreted as an enhanced interest in objects. However, we did not see the same increase in object investigation durations during the object placement paradigms, nor did this occur with ERα agonist PPT. We have also replicated the ability of 50nM 17β-estradiol to enhance object recognition learning in two subsequent experiments (Phan et al, 2012b). Neither of these experiments resulted in increased investigation durations with 17β-estradiol treatment, yet it enhanced object recognition learning. Furthermore, the vehicle group had higher investigation durations than treatment groups during the 17β-estradiol object placement and social recognition paradigms, which did not result in enhanced performance. Therefore, we feel confident in concluding 17β-estradiol does not enhance object recognition performance through increasing investigation durations or interest in objects. This is consistent with observations from other laboratories (Foster 2012).

Interestingly, the hippocampus was able to mediate estrogens’ effects on all three different learning paradigms (object placement, object recognition and social recognition). While the hippocampus is well known to be involved in spatial learning and
memory, it is not necessary for object recognition or social recognition learning (e.g., Squires, et al 2006; Winters, et al 2004). Our studies suggest that although the hippocampus is not necessary for these types of learning, it can still facilitate object and social discrimination performance. We think it is likely that non-genomic estrogenic effects in other brain areas can also contribute to enhancements in these learning paradigms, as well as other types of learning and behavioural responses.

al 1994). The mechanisms that maintain short-term memory and long-term memory appear to be activated in parallel and are at least partly dissociable, since short-term memory can be pharmacologically inhibited without affecting long-term memory and vice versa (reviewed in Izquierdo, et al 2002; Kandel 2001; McGaugh 2000). For example, inhibiting ERK activation in the hippocampus or amygdala does not affect short-term fear conditioning memory (1hr) but inhibits long-term fear conditioning memory (24hrs; Schafe, et al 2000; Trifilieff, et al 2006). To what degree the cellular mechanisms responsible for estrogens’ rapid effects on short-term and long-term memory overlap is unclear at this time.

**Electrophysiology Experiments**

We find that 17β-estradiol and ERα agonist PPT decrease postsynaptic AMPA receptor mediated membrane depolarizations in the hippocampus (Figure 18, 20). Interestingly, we find that the hormone doses producing attenuation of AMPA depolarizations are consistent with the doses that intrahippocampally improved learning for both 17β-estradiol and PPT. None of 17β-estradiol, PPT or DPN affected NMDA receptor mediated membrane depolarizations (Figure 19, 20). The lack of estrogen effects on NMDA receptors is consistent with its role in different phases of learning and memory. While the activation of NMDA receptors during learning acquisition is important for the induction of long-term potentiation and the establishment of long-term memory, it is not necessary for the maintenance or retrieval of short-term memory (~20min of learning; Barker, et al 2006; Winters and Bussey 2005). AMPA receptors however, have been shown to be important for short-term memory, long-term memory and retrieval (Barker, et al 2006; Winters and Bussey 2005). Moreover, while AMPA
receptors have been shown to rapidly cycle into and out of the synapse (within minutes), NMDA receptors do not display these same rapid changes (Lu, et al 2001; Luscher, et al 1999; Rumbaugh, et al 2006). This is consistent with our observations that 17β-estradiol or ER agonists seem to rapidly affect AMPA but not NMDA receptor mediated neurotransmission. Previously, estrogens have been reported to rapidly enhance kainate receptor mediated neurotransmission in cultured female and male rat hippocampal neurons (Gu and Moss 1996; Wong and Moss 1991). One study has found that kainate receptors are necessary for short-term memory, but not long-term memory, which is interestingly the opposite of the role played by NMDA receptors in learning and memory (Barker, et al 2006). Therefore, while we did not examine estrogens’ effects on kainate receptor mediated neurotransmission in this study, estrogens may also affect kainate receptors to non-genomically improve learning and memory.

**Different Roles for ERα and ERβ in Learning and Memory**

One of the interesting results of this study is the different role that is played by ERα and ERβ in estrogens’ non-genomic learning effects. 17β-estradiol was found to rapidly improve learning in general, and this effect was replicated by ERα activation. ERβ activation however enhanced only on object placement learning. This suggests that ERα and ERβ activation have very distinct non-genomic mechanisms of action. In female rat hippocampal sections, 17β-estradiol was shown to increase presynaptic glutamate release from the Schaffer collaterals onto CA1 neurons through ERβ activation, while ERα or GPER activation had no effect (Smejkalova and Woolley 2010). Hence the intrahippocampal delivery of ERβ agonist DPN may have enhanced object
placement performance via presynaptic mechanisms. This interpretation is consistent with the fact that we found no postsynaptic effects of DPN on CA1 hippocampal AMPA or NMDA receptor mediated neurotransmission and that ERβ, not ERα or GPER, increases presynaptic vesicular release (Smejkalova and Woolley 2010). Conversely, ERα and possibly GPER, not ERβ, improve general discrimination learning and induce postsynaptic changes in AMPA receptors and dendritic spine density (Gabor, et al 2012; Phan, et al 2011). Therefore, we suggest that ERα and ERβ may rapidly affect learning through separate mechanisms. ERβ may facilitate object placement learning through affecting presynaptic aspects of glutamate neurotransmission, while ERα may affect general learning processes through postsynaptic mechanisms.

Research is gradually revealing that the non-genomic and genomic actions of estrogens have different effects on neural physiology and do not always produce the same behavioural outcome (e.g., Trainor, et al 2008; Trainor, et al 2007). Contrary to estrogens’ genomic effects on learning and memory which generally imply an important role for ERβ (reviewed in Choleris, et al 2008), here we confirm our previous findings that the rapid effects of 17β-estradiol on learning are ERα mediated. Hence there appear to be dissociable roles for ERα and ERβ in learning and memory via rapid and genomic mechanisms. Similarly, in contrast to estrogens’ non-genomic effects on AMPA receptor mediated transmission, the genomic effects of estrogens have consistently been associated with increases in NMDA receptors, which is thought to be responsible for estrogen-mediated enhancements in long-term memory or memory consolidation (e.g., Jelks, et al 2007; Lewis, et al 2008; Smith and McMahon 2005; Smith and McMahon 2006; Smith, et al 2009; Snyder, et al 2011). Thus, estrogens affect neural physiology
and behaviour through multiple mechanisms, and within natural systems, it is likely that a combination of non-genomic and genomic mechanisms of action occur (reviewed in Vasudevan and Pfaff 2008).

Estrogens and the Creation of Immature Synapses

The decrease in AMPA receptor mediated neurotransmission found in the present study is in agreement with a similar effect previously found in rat cultured cortical neurons. 17β-estradiol application to these neurons for 15-30min caused an internalization of AMPA receptors, and a decrease in AMPA miniature excitatory postsynaptic currents (mEPSC; Srivastava, et al 2008). This estrogen-induced receptor internalization was also associated with simultaneous increases in synapse density. Thus, estrogens appear to rapidly increase the formation of silent or immature synapses (Srivastava, et al 2008). In support of this finding, morphological analysis of estrogens’ rapidly induced dendritic spines in the rat hippocampus reveal an increase in thin and filopodia type, immature spines (Mukai, et al 2007; Murakami, et al 2006), which contain fewer AMPA receptors in the synaptic membrane compared to the mushroom or stubby mature type spines (Kasai, et al 2003). These thin and filopodia spines (as well as silent synapses) show rapid insertion of AMPA receptors upon activation of NMDA receptors, promoting the idea that they are synaptic sites at which new memories can be stored (Kasai, et al 2003; Lu, et al 2001; Luscher, et al 1999; Matsuzaki, et al 2001; Matsuzaki, et al 2004; Rumbaugh, et al 2006).

We have previously shown that systemic doses of 17β-estradiol and PPT producing rapid enhancements in learning also rapidly increased CA1 hippocampal spine density, suggesting spinogenesis may play a role in estrogen-induced learning
enhancements (although we did not examine spine morphotypes; Phan, et al 2011; Phan, et al 2012). The rapid increase in CA1 hippocampal dendritic spines was also shown to be mediated by ERα (Mukai, et al 2007; Murakami, et al 2006; Phan, et al 2011). This coincides with our current findings that the decreases in CA1 hippocampal AMPA receptor mediated membrane depolarizations occurs only in the presence of 17β-estradiol and ERα agonist PPT, but not with ERβ agonist DPN (Mukai, et al 2007; Murakami, et al 2006; Phan, et al 2011). Therefore, 17β-estradiol through ERα both increases dendritic spine density and decreases AMPA receptor membrane depolarizations. Taken together, these data support the idea that estrogens rapidly increase the formation of silent or immature synapses within the CA1 hippocampus via an ERα mechanism. In cultured cortical neurons, stimulating the NMDA receptors of neurons with estrogen-induced silent/immature synapses results in the rapid insertion of AMPA receptors into the membrane surface, increasing AMPA mEPSCs beyond control levels (Srivastava, et al 2008). This data suggests estrogens non-genomically increase the density of immature or silent synapses that may serve as sites for new information storage to facilitate learning. The formation and existence of silent synapses have been proposed to be important for the development and modification of neural circuits (Atwood and Wojtowicz 1999). To what extent the estrogen-mediated production of immature or silent synapses can facilitate learning remains open for future investigations. However, it is reasonable to hypothesize that increases in immature or silent synapses can facilitate learning. In addition, increases in synapse density might lead to wider neuronal interconnectivity and distribution of information for processing.
Conclusions

We show rapid general learning enhancements of 17β-estradiol within the hippocampus that appear to be mediated through ERα. To the best of our knowledge, we present the first experiments drawing strong parallels between the non-genomic effects of estrogens on hippocampal electrophysiology and behavioural learning. We found that the same doses resulting in rapid estrogen-mediated increases in learning attenuated hippocampal AMPA receptor mediated membrane depolarizations. This observation is consistent with the idea that estrogens non-genomically increase the density of silent or immature synapses. These may in turn facilitate learning by providing new sites at which information can be stored.

Acknowledgements

Grants supporting the writing of the paper: NSERC 400212 (EC) and infrastructure grant from CFI 046468 (EC). We would like to thank Enaam Chleilat, Jasmine Aulak and Angela Meersseman for their help in behavioural data collection.
Table 3: A list and description of the behaviours collected and analyzed for learning paradigms (from Phan, et al 2012)

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniff Stimulus</td>
<td>Sniff/Investigation of object or mouse stimulus. Nose twitching and within 1-2mm of stimuli.</td>
</tr>
<tr>
<td>Bite Stimulus</td>
<td>Biting object or mouse stimulus cylinders.</td>
</tr>
<tr>
<td>Sit/Climb on Stimulus</td>
<td>Sitting/climbing on object stimulus with all four paws off the cage floor.</td>
</tr>
<tr>
<td>Dig</td>
<td>Movement of forepaws propelling bedding in posterior direction.</td>
</tr>
<tr>
<td>Bury</td>
<td>Movement of forepaws pushing bedding away from body in anterior direction.</td>
</tr>
<tr>
<td>Horizontal Exploration</td>
<td>Walking, non-stimulus sniffing and exploration of cage.</td>
</tr>
<tr>
<td>Rearing</td>
<td>Both forepaws off the cage floor.</td>
</tr>
<tr>
<td>Self Groom</td>
<td>Grooming with forepaws moving over face and body.</td>
</tr>
<tr>
<td>Inactivity</td>
<td>Includes behaviours such as sit, lay down, freeze and sleep.</td>
</tr>
<tr>
<td>Stereotypy</td>
<td>Strange, repetitive behaviours (&gt;3 repetitions), such as jumps, head shakes, lid chews, etc.</td>
</tr>
</tbody>
</table>
Figure 15: Rapid effects of intrahippocampal 17β-estradiol on learning paradigms.

Asterisks above black bars in A, C, and E represent a significant difference between the investigation percent at habituation (grey bars) versus the investigation percent at test (black bars) for the treatment. (A) 17β-estradiol at 50nM significantly improved object
placement above vehicle treated animals. (B) Vehicle group had a higher total investigation duration than 100nM 17β-estradiol during habituation 1 (H1). (C) 50nM of 17β-estradiol improved object recognition learning. (D) Group treated with 50nM of 17β-estradiol had higher total investigation durations throughout the object recognition paradigm. (E) Mice administered 50nM of 17β-estradiol were successfully able to perform social recognition task. (F) Vehicle control group had higher investigation durations compared to all 17β-estradiol treatment groups. Means and standard error are depicted. *p<0.05, **p<0.01, ***p<0.001.
Figure 16: Rapid effects of intrahippocampal PPT on learning paradigms. Asterisks above black bars in A, C, and E represent a significant difference between the investigation percent at habituation (grey bars) versus the investigation percent at test (black bars) for the treatment. (A) Groups administered PPT at 100nM and 150nM distinguished the displaced object during the object placement paradigm. (C) All groups
administered PPT successfully performed the object recognition task. (E) 100nM of PPT improved social recognition learning. (B,D,F) PPT treatment did not significantly affect total investigation durations in the object placement, object recognition or social recognition experiments. Means and standard error are depicted. *p<0.05, **p<0.01, ***p<0.001.
Figure 17: Rapid effects of intrahippocampal DPN on learning paradigms. Asterisks above black bars in A, C, and E represent a significant difference between the investigation percent at habituation (grey bars) versus the investigation percent at test (black bars) for the treatment. (A) DPN at 100nM improved object placement learning.
(C,E) DPN did not improve object recognition or social recognition learning. (B,D,F) DPN treatment did not significantly affect total investigation durations in the object placement, object recognition and social recognition experiment. Means and standard error are depicted. *p<0.05.
Figure 18: Rapid effects of 17β-estradiol, ERα agonist PPT and ERβ agonist DPN on (S)-AMPA mediated membrane depolarizations in CA1 hippocampal sections. Whole cell current-clamp recordings from CA1 pyramidal neurons. Electrophysiological traces of (S)-AMPA agonist induced membrane depolarizations in 4 neurons (A-D) after 10min of aCSF (left traces; baseline), after 15min of vehicle or hormone application (middle traces), and after 10min of washout with aCSF (right traces). Black lines underneath the tracings indicate the time and duration of 0.4μM (S)-AMPA application. A: traces from a neuron treated with vehicle (aCSF), B: traces from a neuron treated with 50nM of 17β-estradiol, C: traces from a neuron treated with 100nM of ERα agonist PPT, D: traces
from a neuron treated with 100nM of ERβ agonist DPN. The amplitude of (S)-AMPA induced membrane depolarizations were significantly attenuated compared to vehicle controls after 15min of application with 50nM of 17β-estradiol or 100nM of ERα agonist PPT. The amplitude of (S)-AMPA induced membrane depolarizations were unaffected by 15min of 100nM of ERβ agonist DPN treatment. Scales on left: 2mV (vertical axis) and 0.5min (horizontal axis).
Figure 19: Rapid effects of 17β-estradiol, ERα agonist PPT and ERβ agonist DPN on NMDA mediated membrane depolarizations in CA1 hippocampal sections. Whole cell current-clamp recordings from CA1 pyramidal neurons. Electrophysiological traces of NMDA agonist induced membrane depolarizations from 4 neurons (A-D) after 10min of aCSF (left traces; baseline), after 15min of vehicle or hormone application (middle traces), and after 10min of washout with aCSF (right traces). Black lines underneath the tracings indicate the time and duration of 10-15µM NMDA application. A: traces from a neuron treated with vehicle (aCSF), B: traces from a neuron treated with 100nM of 17β-estradiol, C: traces from a neuron treated with 50nM of ERα agonist PPT, D: traces from
a neuron treated with 100nM of ERβ agonist DPN. There were no effects of 17β-estradiol, ERα agonist PPT, or ERβ agonist DPN on the amplitude of NMDA induced membrane depolarizations. Scales on left: 2mV (vertical axis) and 1min (horizontal axis).
Figure 20: Rapid effects of 17β-estradiol, ERα agonist PPT and ERβ agonist DPN on (S)-AMPA and NMDA mediated membrane depolarizations in CA1 hippocampal sections. Whole cell current-clamp recordings from CA1 pyramidal neurons. Percent baseline amplitude of (S)-AMPA and percent baseline amplitude of NMDA mediated depolarizations after 15min of bath applied aCSF (vehicle), 17β-estradiol, PPT or DPN. (A,B) 50nM of 17β-estradiol as well as 100nM and 150nM of PPT significantly attenuated AMPA membrane depolarizations. (C) DPN did not affect AMPA membrane depolarizations. (D,E,F) There was no effect of any hormone treatment on NMDA agonist induced membrane depolarizations. Graphs depict mean and standard error *p<0.05
Supplemental Figure S2: Cannula placements from 17β-estradiol intracranial experiments. Measurements on left indicate position of brain section from Bregma. Images adapted from Paxinos and Franklin 2001.
Supplemental Figure S3: Cannula placements from ERα agonist PPT intracranial experiments. Measurements on left indicate position of brain section from Bregma.

Images adapted from Paxinos and Franklin 2001.
Supplemental Figure S4: Cannula placements from ERβ agonist DPN intracranial experiments. Measurements on left indicate position of brain section from Bregma. Images adapted from Paxinos and Franklin 2001.
CHAPTER 5: General Discussion
This body of work demonstrates for the first time (to our knowledge) that rapid estrogen-mediated learning effects correspond to estrogen-mediated dendritic spine and glutamate neurotransmission effects. Previous articles reporting non-genomic estrogen effects on dendritic spines or electrophysiology are relatively isolated from articles examining the behavioural effects of estrogens on learning, with only theoretical connections between the two fields of study.

Experiments presented within this thesis explore the ability of 17β-estradiol to rapidly affect learning on a time frame corresponding to their non-genomic effects. First, we determined that systemically administered 17β-estradiol and ERα agonist PPT rapidly improved learning (Chapter 2 and 3). The ERβ agonist DPN improved object placement learning, but not object or social recognition (Chapter 3). Second, we determined one site of action for estrogen-induced learning enhancements to be the hippocampus (Chapter 4). Interestingly, these learning effects were also paralleled with increases in dendritic spine density within the CA1 hippocampus, which were remarkably consistent with regards to the timing, estrogen receptor, and drug doses that produced estrogens’ learning effects (Chapter 2 and 3). Furthermore, we conducted experiments to elucidate how estrogens may rapidly affect the electrophysiological properties of CA1 hippocampal neurons. Results from these experiments suggest that there is a decrease in AMPA receptor mediated neural transmission following 17β-estradiol and ERα agonist application to brain sections (Chapter 4). These findings of non-genomic estrogen effects on structural and synaptic plasticity lead us to hypothesize that estrogens promote the development of immature synapses in the hippocampus, which can then be recruited to store new memories when activated through the rapid insertion of AMPA receptors into the synapse
(Srivastava, et al 2008). Based on our results, I propose this may be one basic mechanism through which estrogens can non-genomically improve learning and/or short-term memory.

**New Synapse Formation and Learning and Memory**

One surprising finding from the experiments presented herein and from other published works is that estrogens can increase dendritic spine density in the hippocampus after 15-40 min (Chapter 2 and 3, MacLusky, et al 2005; Mukai, et al 2007; Tsurugizawa, et al 2005). This is a large scale increase in synapses occurring within a short time frame, especially if one considers that on average, each neuron increases potential synaptic connections by 30-50%, and an average neuron possesses ~6000-7000 dendritic connections (Braitenberg and Schuz 1991). If we consider that the widespread synaptic increase occurs within minutes in response to a pharmacological treatment, rather than in response to neural activity, we cannot suppose that these new synapses would form in such a way as to maintain the same circuitry as before the treatment. In fact, a neural system designed in such a rigid fashion would not suit the needs of a structure important for learning and memory and associated plasticity (Trachtenberg, et al 2002).

How is it that this estrogen induced generalized increase in neural connectivity does not result in interference and severe functional impairments? I speculate that these new estrogen-induced synapses must initially be immature synapses, in order to prevent the random formation of neural circuits, or the strengthening of memory traces in a non-specific manner. Upon activation, these immature synapses will be recruited and strengthened to form a memory trace (Matsuzaki, et al 2004; Srivastava, et al 2008). In the absence of neural activity, they may eventually retract after a specific length of time.
or after estrogen levels once again decrease, as would happen naturally across the estrous cycle of female rodents (Woolley and McEwen 1992), in a process conceptually similar to pruning during development (Kantor and Kolodkin 2003).

Both mature, stable synaptic connections and immature, transient synaptic connections have been found to occur naturally in rodent cortical tissue, in the absence of any pharmacological manipulations. In the adult mouse barrel cortex (which processes sensory information from whiskers), long-term repeated in vivo two photon live imaging of neurons labelled with fluorescent protein have shown that most dendritic spines are stable throughout long periods of time (months; Holtmaat, et al 2006; Trachtenberg, et al 2002). In addition, there is a smaller population of new spines that is transient and short lived (tens of minutes) that can, on occasion, become stable, long-lasting spines (Holtmaat, et al 2006; Trachtenberg, et al 2002). Manipulating sensory experience in the mice through whisker trimming results in an increased proportion of stable synaptic connections to become destabilized and disappear, and increases the proportion of transient spines that are converted into long-lasting stable spines (Holtmaat, et al 2006; Trachtenberg, et al 2002). Therefore, these processes are naturally ongoing and neural networks are flexible within the adult nervous system. It seems reasonable to hypothesize that estrogens rapidly activate this same neuronal process, favouring the formation of new synapses to increase neuronal plasticity and promote learning and memory.
Different Mechanisms of Estrogen Action

Several mechanisms of action and several signalling molecules have been implicated to be important for non-genomic estrogen effects on long-term memory consolidation (24-48hrs) within the dorsal hippocampus. Pharmacologically inhibiting NMDA receptors, PKA, PI3K, ERK, protein synthesis, as well as histone acetylation and DNA methylation in the dorsal hippocampus have been shown to inhibit estrogen-mediated enhancements in long-term object recognition memory (Fan, et al 2010; Fernandez, et al 2008; Fortress, et al 2013; Lewis, et al 2008; Zhao, et al 2010).

However, the cellular mechanisms underlying short-term memory and long-term memory appear to be parallel and independent (rather than organized serially; reviewed in Izquierdo, et al 2002; Kandel 2001; McGaugh 2000). Since the learning paradigms used within this thesis relies on short-term memory, the cellular mechanisms behind the estrogen-induced learning improvements reported here are likely different than those for estrogens’ long-term memory effects. It is possible to speculate on a few candidate molecules, based on their known involvement in hippocampal short-term memory.

protein (CREB; Bailey, et al 2000), protein kinase C (PKC) and protein kinase G (PKG)(Izquierdo, et al 2000) inhibition do not seem to affect short-term learning. Whether PKA activity within the hippocampus is important for short-term memory, is currently unclear given conflicting results of several studies (reviewed in Izquierdo, et al 2002, but see Abel, et al 1997). In view of these findings, I recommend future studies into the cellular mechanisms of estrogens rapid effects on short-term memory in the hippocampus focus on AMPA receptors, mGluR, BDNF, PI3K, and possibly PKA, since we (and others) have found that estrogens rapidly affect AMPA neurotransmission (Srivastava, et al 2008), affect mGluR (Huang and Woolley 2012, and reviewed in Micevych and Christensen 2012), interact or have similarities with BDNF (reviewed in Srivastava, et al 2013), and increase PI3K (Fan, et al 2010) and PKA (Lewis, et al 2008) activity.

From the body of literature on the effects of estrogens, it is clear that they have a very wide range of action. Even within one type of tissue, neural tissue, the effects of estrogens are highly variable, dependent on receptor subtype, the brain regions in which they are expressed, and the pathway through which they act: genomic or non-genomic. Sometimes, estrogens acting through different pathways and receptors work together to foster a specific behaviour (e.g., sexual behaviour Vasudevan, et al 2005), while other times they can work in opposition to one another (e.g., aggression, Trainor, et al 2008; Trainor, et al 2007). Hence the variability of estrogen effects may provide a system that is highly capable of evolutionary and experience dependent changes. The repurposing of existing systems for different functions is a common theme in evolution (Goldman, et al 2012). Moreover, we can hypothesize that we would expect to see a wide range of
estrogen effects within the central nervous system, since this is a tissue that is, by nature, necessarily highly plastic and amenable to experience dependent changes.

The rapid behavioural effects of estrogens are particularly interesting, especially in the context of the discovery that brain tissue is capable of synthesizing estrogens de novo. It has been suggested that the rise in plasma estradiol that occurs during the estrus cycle (at the proestrus stage) may be too slow to be responsible for the non-genomic estrogen effects on behaviour (Cornil, et al 2006c). It has also been reported that neural activity (including glutamate activity) can quickly, within minutes, modulate local aromatase activity, the enzyme which is responsible for the conversion of testosterone into estrogen during the last step of the steroidogenic pathway (Balthazart, et al 2006; Hojo, et al 2004). Hence the local concentrations of estrogens within the brain can be quickly and significantly manipulated by neural activity, which may then promote non-genomic behavioural estrogen effects. Therefore, it appears possible that the local brain synthesis of estrogens may be an important driver of the non-genomic effects of estrogens on behaviour (Cornil, et al 2006c). The reports of local brain synthesis and its link with the rapid effects of estrogens on behaviour provide an intriguing avenue to pursue in future studies.

**Significance and Applications of Studying Estrogens and Learning**

Human and animal studies consistently demonstrate individuals having undergone natural or surgical menopause have impaired cognitive functions compared to individuals with intact, functioning ovaries (reviewed in Choleris, et al 2008; Farrag, et al 2002). When estrogens are replaced in these individuals, their effects on learning and memory systems are largely beneficial (reviewed in Choleris, et al 2008; Duff and Hampson 2000;
Sherwin 2006), although with some negative findings. In particular, the largest randomized clinical trial of hormone replacement therapy (HRT; Women’s Health Initiative study) concluded HRT provided no beneficial cognitive effects, and their risks (e.g. uterine or breast cancers) outweighed any benefits of treatment (e.g., Klein and Rapp 2004). However, problems with the HRT used in the study such as high drug doses, the use of synthetic, non-endogenous compounds in particular, age of women, and the number of years women were postmenopausal before the start of HRT (Morrison, et al 2006; Schumacher, et al 2003), raise concerns about whether this conclusion may have been premature. By researching how estrogens affect learning and memory, we can develop effective HRTs, or find new treatment options for post-menopausal cognitive decline, while avoiding the serious negative side effects associated with HRT.

The results of the studies presented herein have several implications for HRT and the treatment of post-menopausal cognitive decline. First, we demonstrate estrogen-mediated learning enhancements and synapse increases have an inverted U-shaped dose response curve. Only physiological doses of 17β-estradiol appear to be effective, and similarly, a narrow ER agonist dose range was effective. This suggests that any beneficial effect of clinical estrogen treatment will be highly dose-dependent, and treatments should be designed to produce physiological plasma levels of estradiol in postmenopausal women. High doses of estrogen treatment will likely be ineffective for cognitive enhancement, and may in fact unnecessarily increase risks of serious negative side effects, such as those observed in the WHI study. Secondly, there has been optimism that the cognitive benefits of HRT can be achieved through ERβ agonists, without increasing the risks of uterine or breast cancers, since these tissues do not express ERβ (Choleris, et
However, our results indicate that estrogens rapidly enhance learning primarily through ERα, not ERβ, suggesting that these receptors may play different roles during different phases of memory processing. Thus, we would caution that the cognitive benefits of an ERβ agonist HRT may be limited.

Another possibility for the treatment of post-menopausal cognitive decline can be to stimulate the natural process of local estrogen production, instead of administering exogenous estrogens. The vitamin A metabolite retinoic acid stimulates production of estrogens, including in hippocampal tissues (Munetsuna, et al 2009). Future experiments with a direct clinical application would be to examine whether retinoic acid delivered intrahippocampally or orally is able to improve learning in ovariectomized mice. If retinoic acid improves learning via an enhancement of local estrogen synthesis, it may lead to a novel treatment option for post-menopausal cognitive decline that: 1) allows women to avoid the negative side effects of systemic HRT, and 2) is simple and cost effective, since retinoic acid can be supplemented through one’s diet. If successful, this research can potentially translate into a promising treatment for post-menopausal cognitive decline.
CHAPTER 6: References


Frye CA and Rhodes ME (2002). Enhancing effects of estrogen on inhibitory avoidance performance may be in part independent of intracellular estrogen receptors in the hippocampus. *Brain Res* **956**: 285-293.


as well as endocrine disrupters in hippocampal neurons. *Brain Res Rev* 57: 363-375.


Smith CC and McMahon LL (2005). Estrogen-induced increase in the magnitude of long-term potentiation occurs only when the ratio of NMDA transmission to AMPA transmission is increased. *Journal of Neuroscience* **25**: 7780-7791.


