Estrogens regulate the synaptic proteome in a sexually dimorphic manner through local protein synthesis

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ESTROGENS REGULATE
THE SYNAPTIC PROTEOME
IN A SEXUALLY DIMORPHIC
MANNER THROUGH LOCAL
PROTEIN SYNTHESIS

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ABSTRACT

Estrogens, particularly its biologically active form 17β-estradiol (estradiol), have repeatedly been illustrated to have long-lasting influences over cognitive function and behaviour, which is believed to be, in part, driven by estrogenic regulation of synaptic plasticity. Specifically, estrogens have been shown to rapidly, within minutes, regulate dendritic spine dynamics, and shape synapse structure and function. Critically, the rapid effects of estrogens on synapses are mediated by specific signalling pathways, which have been shown to be required for estrogenic-facilitation of learning and memory. More recently, emerging evidence indicates that estrogens regulate learning and memory through the modulation of local protein synthesis; the ability to produce nascent proteins without the need for gene transcription. However, the molecular and cellular mechanisms that underlie estrogen’s ability to regulate local protein synthesis and furthermore, whether such a mechanism can ultimately impact synaptic function in both male and female brains is currently unknown. The role of acute estrogen signalling in regulating local protein synthesis within the hippocampus of males and females was investigated in this thesis.

Employing a combination of Surface Sensing of Translation (SUnSET) and fluorescent non-canonical amino acid tagging (FUNCAT) assays, estradiol was found to increase protein synthesis within 2 hour estradiol treatment in male and ovariectomised (OVX) hippocampal slices. This was mediated by two different signalling pathways in both sexes. Interestingly, in concert with this increased local translation, there was an increase in a subset of synaptic proteins and dendritic spines observed within the same time-frame in both sexes. These proteins are targeted to dendritic spines in the primary neuronal model. This study proposes a novel mode of estrogen signaling that could result in long-lasting changes in neuronal circuitry and cognitive function. Further research is warranted to test the beneficial effects of estradiol in the context of a local protein synthesis mechanism being therapeutic.
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4 years ago, I was sitting in my supervisor’s office and before the confirmation of acceptance, he said: ‘This is the hardest thing you’ll ever do’. It is not until a few months ago I realised what that meant. Yes, it was the hardest thing I’ve ever done but several people are to thank for making this journey what it was.

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STATEMENT OF WORK COMPLETED

All writing and data presented within this thesis was the result of work completed by myself unless otherwise stated.

Chapter 2: The spine representation images from Figure 2.5J-L were taken from an experiment conducted and imaged by Iain Watson. Iain Watson also designed and provided Figure 2.7.

Chapter 3: Hannah Creeney carried out the western blotting of particular targets, which contributed to the representation image of data presented, these are figures: 3.2D, 3.3A+B, 3.4A+D. In these cases, the lysates were prepared by myself, western blotting by Hannah Creeney, and subsequent quantification and analysis of all the blots run, by me.

Chapter 4: Hannah Creeney carried out the western blotting in Figure 4.6A, which contributed to the representation image of data presented. The lysates were prepared by myself, western blotting by Hannah Creeney, and subsequent quantification and analysis by me.

Chapter 5: Dr. Rodrigo Duarte designed the primers, ran the qPCR, and performed the analysis for the Figure 5.3C-E. For this experiment, I carried out the pharmacological treatment and RNA isolation under the supervision of Dr. Rodrigo Duarte. Iain Watson imaged the third biological replicate for the data that contributed to Figures 5.8-10 whereby the quantification and analysis was performed by me.

Appendix: Hannah Creeney carried out the western blotting in Appendix 1B, which contributed to the representation image of data presented and the lysates were prepared by myself. Appendix 3A was imaged by myself with the help of Laura Sichlinger from an experiment conducted by Hannah Creeney. The image was processed by myself.
## CONTENTS

**Abstract** ........................................................................................................................................... 2

**Acknowledgements** .......................................................................................................................... 3

**Statement of work completed** ........................................................................................................ 5

**List of figures** ..................................................................................................................................... 11

**List of tables** ....................................................................................................................................... 14

**Abbreviations** ..................................................................................................................................... 15

**Chapter 1: Introduction** ................................................................................................................... 19

**Overview** ........................................................................................................................................... 19

**Estrogen signalling in the brain** ......................................................................................................... 21

  - Classical vs. rapid signalling ........................................................................................................... 21
  - Estrogen synthesis in the hippocampus............................................................................................ 22
    - Localisation of Aromatase .............................................................................................................. 23
    - Role in plasticity ............................................................................................................................. 24
  - Estrogen receptors in the hippocampus ............................................................................................. 24
    - ERs and their localisation ............................................................................................................... 24
    - ER signalling .................................................................................................................................. 26

**Modulation of cognition and underlying mechanisms** ..................................................................... 28

  - Hippocampal memory ....................................................................................................................... 28
    - Acute estradiol treatment on memory – acquisition studies in OVX female rodents .................. 29
    - Acute estradiol treatment on memory – consolidation studies in OVX female rodents ............ 30
    - Memory studies in male rodents .................................................................................................... 31
  - Molecular mechanisms underlying memory enhancement ............................................................. 32
    - ERs implicated in enhancing hippocampal memory ...................................................................... 32
    - Cell signalling cascades regulating estradiol-induced memory consolidation in rodents ........ 33
    - Remodeling of synaptic structure and function .............................................................................. 34
  - A role for local protein synthesis ..................................................................................................... 36
  - A consideration for sex differences .................................................................................................. 37

**Local protein synthesis** .................................................................................................................. 37

  - Local vs. global protein synthesis .................................................................................................. 37
Protein synthesis machinery at dendrites & axons .................................. 39
Dendrites .................................................................................................. 39
Axons ...................................................................................................... 41
Role in synaptic plasticity and memory .................................................. 42
Regulation of local protein synthesis .................................................... 43
Signalling pathways .............................................................................. 43
MicroRNAs ............................................................................................ 45
Contribution of pre-synaptic structures .................................................. 46
Aims and hypotheses ............................................................................. 47

Chapter 2: Materials and methods ....................................................... 48

Animals .................................................................................................... 48

Primary neuronal culture ...................................................................... 49
Acute slice preparation ......................................................................... 49
Plasmid DNA transfection ..................................................................... 50
Pharmacological treatments ................................................................... 50

Lysate preparation ................................................................................ 51
Whole cell lysates ................................................................................ 51
Crude synaptosomal preparation ......................................................... 52
Western blotting ................................................................................... 53
Quantification of western blots ............................................................. 53

RNA isolation ....................................................................................... 54
RNA extraction ..................................................................................... 54
cDNA synthesis ................................................................................... 55
Quantitative reverse transcription polymerase chain reaction (RT-qPCR) . 55

Immunocytochemistry .......................................................................... 56
Detection of protein synthesis ............................................................... 57
Surface Sensing of Translation (SUnSET) ............................................. 57
Fluorescent noncanonical amino acid tagging (FUNCAT) ................... 59

Microscopy and image acquisitions ...................................................... 61
Epifluorescence imaging and confocal microscopy ............................... 61
Structural Illumination Microscopy (SIM) ............................................ 62
Quantitative analysis of dendritic spine morphology and
immunofluorescence ........................................................................... 63
Analysis of new protein expression using FUNCAT ............................ 65
Analysis of puncta co-localisation ....................................................... 66
Chapter 3: Acute estrogen signalling regulates synaptic protein expression in a sexually dimorphic manner

Summary .......................................................................................................................... 73
Introduction ...................................................................................................................... 73
Results .............................................................................................................................. 77
Estradiol increases the expression of key excitatory post-synaptic proteins in both male and OVX female hippocampus ................................................. 77
Estradiol induces a sex specific difference in the expression of post-synaptic inhibitory proteins ................................................................. 82
Excitatory pre-synaptic proteins are differentially altered in response to estradiol ................................................................. 84
Sex specific expression difference is observed in specific pre-synaptic inhibitory proteins ................................................................. 86
Discussion ......................................................................................................................... 88
Summary of results .......................................................................................................... 88
Estradiol mediates synaptic plasticity predominantly through a post-synaptic mechanism in the hippocampus ......................................................... 88
Contribution of an estradiol-mediated pre-synaptic mechanism ......................... 91
Estradiol induces expression changes in synaptic proteins in a sexually dimorphic manner ................................................................. 93
Conclusions ....................................................................................................................... 94

Chapter 4: Estrogen increases local protein synthesis in a sexually dimorphic manner

Summary .......................................................................................................................... 97
Introduction ...................................................................................................................... 98
Results .............................................................................................................................. 100
Estradiol increases the rate of protein synthesis in hippocampal slices ... 100
Estradiol-induced increase in protein synthesis in dependent on a translation mechanism ......................................................................... 103
mTOR is required for estradiol-dependent increase in protein synthesis in the male, but not OVX female, hippocampus ................................................. 107
Estradiol acutely increases protein translation in crude synaptosomal fractions ................................................................. 111
Newly synthesized proteins are found in dendritic spines and along dendrites following 2 hour estradiol treatment ................ 113
New proteins are synthesized independently of gene transcription through the mTOR signalling pathway in dendritic regions in response to estradiol ........................................................................................................................................ 116
Estradiol induces spinogenesis within the same time-frame .......... 121

Discussion ......................................................................................................................................................................................... 125
Summary of results ........................................................................................................................................................................ 125
Estradiol increases protein synthesis, independent of gene transcription in hippocampal neurons ........................................ 125
Distinct signalling mechanisms mediate local protein synthesis between sexes ......................................................................................................................... 127
Newly synthesised proteins are found in larger dendritic spines .... 129
Conclusions ......................................................................................................................................................................................... 130

Chapter 5: Estrogen increases synaptic PSD-95 and GluN2B in local protein synthesis dependent manner .......................... 131
Summary ......................................................................................................................................................................................... 131
Introduction ...................................................................................................................................................................................... 132
Results ............................................................................................................................................................................................ 134
Estradiol increases expression of PSD-95 and GluN2B in a translation dependent manner .......................................................... 134
Estradiol increases PSD-95 and GluN2B expression in crude synaptosomal fractions while having no effect on mRNA levels in primary hippocampal neurons ........................................................................................................................................ 139
PSD-95 and GluN2B are increased specifically within dendritic spines and along dendrites following estradiol treatment .......... 142
mTOR is required for estradiol-dependent increase of PSD-95 in both and OVX females ......................................................................................................................................................................................... 145
Estradiol increases GluN2B expression independent of mTOR in OVX females but not males ............................................ 147
RPS6 is phosphorylated in males but not OVX females ............... 148
Estradiol rapidly increases the rate of translation along dendrites and juxtaposing synaptic regions ................................................................. 151
RPS10 is increased along dendrites and within spine regions ............ 154
Co-localisation of puromycin-tagged nascent proteins and RPS10 is increased....................................................................................... 157

Discussion ........................................................................................................ 159
Summary of results ......................................................................................... 159
Estradiol increases expression of PSD-95 and GluN2B in a local protein synthesis dependent manner at dendritic spines ......................... 159
mTOR is required for estradiol to increase the expression of specific proteins ......................................................................................... 162
Conclusions...................................................................................................... 164

Chapter 6: General discussion ..................................................................... 165
Summary of findings ...................................................................................... 165
Implications .................................................................................................... 168
  A novel mechanism to regulate synaptic plasicity .................................. 168
  Relevance to neurological diseases ............................................................ 170
Limitations and future directions ................................................................. 172
  OVX female rodent models ........................................................................ 172
  Alternative methods to investigate local protein synthesis ................ 173
  Other signalling cascades implicated in estradiol-mediated protein synthesis ......................................................................................... 175
  Role of ERs .................................................................................................. 176
  Estrogenic signalling within the cerebral cortex ....................................... 177
Concluding remarks ........................................................................................ 177

References .................................................................................................... 178
Appendices .................................................................................................... 204
### LIST OF FIGURES

#### Chapter 1

| Figure 1.1 | Rapid estrogen membrane-signalling | 28 |
| Figure 1.2 | Estrogen rapidly enhances memory in rodents | 32 |
| Figure 1.3 | The complexity of the synaptic proteome | 38 |
| Figure 1.4 | The population of mRNA transcripts found in synaptic compartments | 41 |

#### Chapter 2

| Figure 2.1 | Schematic of pharmacological treatment timeline in primary | 51 |
| Figure 2.2 | Schematic of the Surface Sensing of Translation (SUnSET) assay to measure the rate of protein translation | 58 |
| Figure 2.3 | Schematic of puromycin (SUnSET assay) treatment timeline in primary rat neurons and acute slice preparations from mice within this study | 58 |
| Figure 2.4 | Schematic of azidohomoalanine (AHA) treatment and subsequent fluorescent noncanonical amino acid tagging labelling (FUNCT) protocol timeline in primary rat neurons within this study | 61 |
| Figure 2.5 | Analysis of dendritic spine morphology in cultured primary neurons using Metamorph | 64 |
| Figure 2.6 | Analysis parameters for AHA-tagged newly synthesised proteins in cultured primary neurons | 65 |
| Figure 2.7 | Analysis parameters for determining spine and dendritic regions for puncta analysis in MAP2 positive neurons | 67 |

#### Chapter 3

| Figure 3.1 | Estradiol acutely increases PSD-95 and GluA1 expression in the male and OVX female hippocampus | 80 |
| Figure 3.2 | NMDAR subunits are increased in the male and OVX female hippocampus following 2 hour estradiol treatment | 81 |
| Figure 3.3 | Estradiol differentially regulates key inhibitory post-synaptic proteins in the male and OVX female hippocampus | 83 |
Figure 3.4. Estradiol increases a subset of excitatory pre-synaptic proteins in a sex dependent manner after 2 hours ........................................................................................................85

Figure 3.5. Key inhibitory synaptic proteins are differentially regulated by acute estradiol treatment ........................................................................................................87

Chapter 4

Figure 4.1 Estradiol increases protein synthesis in the male and OVX female hippocampus .....................................................................................................................102

Figure 4.2 Estradiol-mediated increase in protein synthesis is inhibited in the presence of anisomycin ........................................................................................................105

Figure 4.3 Estradiol increases protein synthesis independent of gene transcription. .................................................................................................................................106

Figure 4.4 mTOR is required for estradiol-mediated protein synthesis in the male hippocampus ..............................................................................................................108

Figure 4.5 Activation of mTOR kinase signalling pathway following 2 hour estradiol treatment ..............................................................................................................110

Figure 4.6 Estradiol acutely increases protein synthesis in crude synaptosomal fractions from primary hippocampal neurons ........................................................................112

Figure 4.7 AHA-tagged newly synthesised proteins are increased along dendrites and within dendritic spines. ..........................................................................................115

Figure 4.8 Anisomycin inhibits the increase of nascent proteins along dendrites and within dendritic spines in estradiol treated neurons ..............................................118

Figure 4.9 Estradiol treated neurons exhibit increased nascent proteins in dendritic and spine regions in the presence of actinomycin D ..............................................119

Figure 4.10 mTOR is required for estradiol to increase new proteins along dendrites and spines .................................................................................................120

Figure 4.11 Estradiol increases spine number and area within 2 hours .......122

Figure 4.12 New proteins are localised to larger dendritic spines in estradiol treated neurons ..............................................................................................................124
Chapter 5

**Figure 5.1** Estradiol-mediated increase in PSD-95 and GluN2B expression is inhibited in the presence of anisomycin in both male and OVX female hippocampus .......................................................... 137

**Figure 5.2** Actinomycin D does not affect estradiol-mediated increase of PSD-95 and GluN2B expression in the male and OVX female hippocampus .............................................. 138

**Figure 5.3** Estradiol increases PSD-95 and GluN2B expression in crude synaptosomal fractions but doesn’t affect the respective mRNA expression in primary hippocampal neurons.

.............................................................................................................. 141

**Figure 5.4** PSD-95 is increased along dendrite and within dendritic spines in primary hippocampal neurons.

.............................................................................................................. 143

**Figure 5.5** GluN2B is increased within dendritic spines but not extrasynaptically in primary hippocampal neurons.

.............................................................................................................. 144

**Figure 5.6** mTOR is required for estradiol-mediated increase in PSD-95 expression in the male and OVX female hippocampus but mTOR is required for estradiol-mediated increase in GluN2B only in the male but not OVX female hippocampus.

.............................................................................................................. 146

**Figure 5.7** Estradiol increases phosphorylation of ribosomal protein S6 (RPS6) in the male, but not OVX female hippocampus ................................................................. 150

**Figure 5.8** Estradiol increases puromycin-tagged elongating proteins, representing active translation sites, along dendrites and within dendritic spines in primary hippocampal neurons.

.............................................................................................................. 153

**Figure 5.9** Ribosomal protein S10 (RPS10) is increased along dendrites and within spine regions following 2 hour estradiol treatment in primary hippocampal neurons.

.............................................................................................................. 156

**Figure 5.10** Estradiol increases co-localisation of RPS10 with puromycin along dendrites and within spine regions following 2 hour estradiol treatment in primary hippocampal neurons ................................................................. 158
LIST OF TABLES

Chapter 2

Table 2.1 Composition of common solutions ..........................................................69
Table 2.2 Drug compounds, and their final concentrations, used in pharmacological treatments in both primary neurons and acute slices ..........69
Table 2.3 List of primary antibodies used within the investigation ......................70
Table 2.4 List of secondary antibodies used within the investigation ...............72
Table 2.5 Primer sequences used within the investigation ....................................72

Chapter 3

Table 3.1 Summary of synaptic protein expression change in male and OVX female acute hippocampal slices following a 2 hour estradiol treatment ..........96
ABBREVIATIONS

4EBP1 Eukaryotic initiation factor 4E-binding protein 1
aCSF Artificial cerebrospinal fluid
AHA Azidohomoalanine
AEBSF 4-benzenesulfonul fluoride hydrochloride
Ago Argonaute
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type receptors
APV (2R)-Amino-5-Phosphovaleric Acid
Aromatase Aromatase cytochrome P450
BCA Bicinchoninic acid
BCP 1-bromo-3-phenolpropane
BDNF Brain-derived neurotrophic factor
BSA Bovine serum albumin
CAMKIIα Calcium/calmodulin-dependent protein kinase type II alpha
CHO Chinese Hamster Ovary cells
d-N108-15 differentiated neuroblastoma cell line NG108-15
DAPI 4′6-diamidino-2-phenylindole
DHPG (RS)-3,5-Dihydroxyphenylglycine
DISC1 Disrupted in schizophrenia 1
DIV Days in vitro
DMEM Dulbecco’s Modified Eagle’s Medium
DMSO Dimethyl sulfoxide
DPN 2,3-bis(4-Hydroxyphenyl)-propionitrile
DTT Dithiothreitol
E-LTP Early phase long-term potentiation
E/I Excitatory/inhibitory balance
E2 17β-estradiol; estradiol
eEF2 Eukaryotic elongation factor 2
eGFP Enhanced green fluorescent protein
eIF2 Eukaryotic initiation factor 2
eIF4E Eukaryotic translation initiation factor 4E
EPSC excitatory postsynaptic current
ERα Estrogen receptor alpha
ERβ Estrogen receptor beta
ERE Estrogen binding element
ERK Extracellular signal-regulated kinase 1/2
FA Formaldehyde
FMR1 Fragile X mental retardation 1 gene
FMRP Fragile X mental retardation protein
FUNCAT Fluorescent non-canonical amino acid tagging
FXS Fragile X Syndrome
G1 rel-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone
GABA Gamma-aminobutyric acid
GABAR Gamma-aminobutyric acid type receptor
GABA α1 Gamma-aminobutyric acid type receptor subtype alpha 1
GAD-65/67 Glutamatic acid decarboxylase 65/67
GDX Gonadectomised
GFP Green fluorescent protein
GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type receptor 1
GluN1 N-methyl-d-aspartic acid type receptor 1
GluN2A N-methyl-d-aspartic acid type receptor 2A
GluN2B N-methyl-d-aspartic acid type receptor 2B
GPER1 G-protein coupled estrogen receptor
Gq-mER Gq protein-coupled membrane-initiated estrogen receptor
HBSS Hank’s balanced salt solution
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hiPSCs Human induced pluripotent stem cells
HPG homopropargylglycine
ICC Immunocytochemistry
iGluRs Ionotropic glutamate receptors
KO Knockout (animal model)
L-LTP Late phase long-term potentiation
LF2K Lipofectamine 2000
LTD Long-term depression
LTP Long-term potentiation
mIPSCs Miniature inhibitory postsynaptic currents
miRNA MicroRNAs
mGluR Metabotropic glutamate receptor
mRNA Messenger RNA
mTOR Mammalian target of rapamycin
N.A. Numerical Aperture
NFT Neurofibrillary tangles
NGS Normal Goat Serum
Nlgn1 Neuroligin 1
Nlgn2 Neuroligin 2
NMDA N-methyl-d-aspartic acid
NMDAR N-methyl-d-aspartic acid type receptor
NT-3 Neurotrophin-3
OP Object placement task/memory
OR Object recognition task/memory
OVX ovariectomised
p-RPS6 Phosphorylation of RPS6
P13/Akt Phosphatidylinositol 3-kinase/Akt
PBS Phosphate-buffered saline
Pen/strep Penicillin:streptomycin
PDL Poly-D-Lysine
PFA Paraformaldehyde
PPT 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol
Pre-mRNA Precursor mRNA
Pri-mRNA Primary mRNA
PSD Postsynaptic density
PSD-95 Postsynaptic density protein 95
PVDF Polyvinylidene difluoride
RBP RNA binding protein
RISC RNA-induced silencing complex
RNAselll Nuclear ribonuclease III
ROI Region of interest
RPS6 Ribosomal protein S6
RPS10 Ribosomal protein S10
rRNA Ribosomal RNA
RT Room temperature
RT-qPCR Quantitative reverse transcription polymerase chain reaction
S6K p70 ribosomal S6 kinase
SEM Standard error of mean
SIM Structured Illumination Microscopy
sIPSCs Spontaneous inhibitory postsynaptic currents
SNAP 25 Synaptosomal associated protein 25
SIII Superscript III™ Reverse Transcriptase
SUnSET Surface sensing of translation
SUnSET-ICC Surface sensing of translation by western blotting
SUnSET-WB Surface sensing of translation by immunocytochemistry
SV2A Synaptic vesicle protein 2A
T1 Training period (OR/OP memory tasks)
T2 Testing period (OR/OP memory tasks)
TBS-T Tris-buffered saline, Tween 20
TIFF Tagged Image File Format
TRAP Translating Ribosome Affinity Purification
tRNA Transfer RNA
UTR Untranslated region
VGAT Vesicular inhibitory amino acid transporter
vGlut1 Vesicular glutamate transporter 1
WAY 20070 7-Bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol
There is compelling evidence that the regulation of structure and function of neuronal circuits is an essential component of normal cognitive function and behaviour. The general consensus is that neuronal circuits, post initial formation, still remain plastic to a certain extent during adulthood. This allows different morphological modifications to occur in response to environmental and extracellular stimuli during adulthood. For instance, agents such as steroid hormones are able to trigger alterations in distinctive elements of the neuronal circuity through the initiation of different signalling cascades and neuronal processes. There is mounting evidence illustrating that alterations in the neuronal circuity seems to correlate with alterations in distinct processes such as learning and memory, and behaviour.

Steroid hormones such as estrogens have been reported to influence nervous system development and function (Losel et al., 2003). They have been implicated in numerous physiological functions such as reproductive, development, cardiovascular and neuronal function (Brinton, 2009; McEwen and Alves, 1999). One of the most studied effects of estrogens in the brain is its ability to influence cognition. The enhancing effects of estrogens has been reported in rodent and non-human primate models and to a lesser extent humans (Sellers et al. 2015a). Estrogens have also been suggested to have beneficial effects in disease both in protection against pathology and potentially as a restorative agent (Sellers et al., 2015a; Srivastava et al., 2013). However, a large clinical trial, the Women’s Health Initiative, found no benefits on cognition from hormone therapy and, instead reported a decline in cognitive
function and increased risk of dementia and stroke (Espeland et al., 2004; Shumaker et al., 2003, 2004). This study was widely criticised due to: the use of 65 years or older, women who were at least 15 years post-menopause; the variability in their health; and the use of conjugated equine estrogens and synthetic progesterone within the trial (Brinton, 2005). In support, studies have proposed a ‘window of opportunity’ for when the brain is still responsive to estrogens following menopause or surgical removal of ovaries (Singh et al., 2013). In support of this, other studies have reported positive effects of estrogens on memory in female humans (Sherwin and McGill, 2003). Recently, work from our group has begun to investigate whether estrogens can induce cellular effects associated with enhancement of cognition, in human neurons generated from induced pluripotent stem cells (hiPSCs) (Shum et al., 2015). Consistent with the prediction that estrogens can positively modulate cognition in humans, estrogens have shown to regulate cellular parameters such as structural plasticity in hiPSC-neurons (Shum & Srivastava, personal communication). Therefore, together with the beneficial effects of estradiol reported on rodent and non-human primate models, and recently in hiPSC-models from our lab, there is increased interest for further research on understanding the role of estrogens on cognitive functioning and behaviour.

Classically, estrogens, and in particular the biologically active form 17β-estradiol (estradiol; E2), have been demonstrated to exert their effects on the brain through gene transcription over several hours to days (Srivastava et al., 2013). However, it is now greatly appreciated that they can also facilitate rapid effects that manifest within minutes to hours (Frick and Kim, 2018; Luine, 2015; Sellers et al., 2015a; Srivastava et al., 2013). Much recent research has delved in understanding the underlying molecular mechanisms of these rapid effects on the functioning of the hippocampus and much of the work from our lab has focussed on the cortex. These rapid estrogenic actions have been shown to mediate different neuronal functions such as synaptic plasticity, cognition and neuroprotection (Srivastava et al., 2013). Countless rodent studies have exemplified the significance of rapid estrogen-induced modulatory effects on cognitive augmentation, including memory processing and social behaviours (Choleris et al., 2012; Frick and Kim, 2018; Luine et al., 2018). The long-lasting influences over cognitive function and behaviour is believed to be in part driven
by estrogenic regulation of synaptic plasticity (Srivastava and Penzes, 2011). The underlying molecular and cellular mechanisms mediating this plasticity have yet to be fully elucidated. Multiple lines of evidence have demonstrated that estradiol can rapidly influence protein translation machinery and signalling pathways involved in protein synthesis in neurons. However, the mechanisms linking rapid estrogenic signaling with protein synthesis, and the outcome of engaging this mechanism is currently unknown. The following sections will review the influence of estradiol on synaptic plasticity in the brain. Following this, evidence for local protein synthesis along dendrites of neuronal cells will be discussed. Finally, evidence for a role for local protein synthesis in estrogenic signaling will be presented.

ESTROGEN SIGNALLING IN THE BRAIN

Classical vs. rapid signalling

A series of landmark papers first identified that estradiol could influence the neuronal architecture. The rat estrous cycle was found to mediate fluctuations in dendritic spines (Woolley et al., 1990) and synapse density (Woolley and McEwen, 1992) in the CA1 region of the hippocampus. Dendritic spines are small actin-rich protrusions that decorate the dendrites of neuronal cells and house post-synaptic densities (PSDs) (Chubykin et al., 2007; Harris and Stevens, 1989). PSDs consist of a large number of key proteins that synchronise to form functional excitatory synapses - the point of communication between two neurons (McAllister, 2007). Ovariectomy\(^1\) (OVX)-induced loss of dendritic spines was found to be rescued by chronic estradiol treatment in the CA1 hippocampus (Gould et al., 1990). Studies that followed showed that: dendritic spine numbers were highest during the proestrous phase where estradiol levels are higher and lowest during estrous phase where estradiol levels are lower (Woolley et al., 1990); synapse density followed the same pattern (Woolley and McEwen, 1992); estradiol treatment increased synapse density (Woolley and McEwen, 1992); and estradiol-mediated increase in dendritic spines was dependent on \(N\)-methyl-D-aspartate receptor (NMDAR)

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\(^1\) The surgical removal of one or both ovaries.
activation (Woolley and McEwen, 1994). These observations were then thought to be dependent on gonadally-driven estradiol acting on the brain. Current research has juxtaposed the classical views of estrogens influencing plasticity over several hours to days. It is now greatly appreciated that estrogens can rapidly facilitate plasticity, within minutes to hours through a ‘non-genomic’ mechanism (Srivastava et al., 2013), which is reviewed later. This rapid estradiol-mediated modulation is supported by compelling research challenging the classical dogma that the brain is targeted by gonadally-driven estrogens. There is accumulating evidence illustrating the presence of estrogen receptors (ERs) at extra-nuclear sites such as synapses and along dendritic shafts, in addition to the nucleus. Moreover, estradiol can also be synthesised de novo in the brain (Frick, 2015; Sellers et al., 2015a; Srivastava et al., 2013). It is thus, thought that estradiol synthesised de novo in the brain may locally control synaptic changes within a rapid time-frame in the brain.

**Estradiol synthesis in the hippocampus**

The rapid effects of estradiol in the brain have been suggested to be mediated by locally synthesised estradiol (Cornil et al., 2012; Srivastava and Penzes, 2011). The biosynthesis of estrogens both peripherally and within the brain depend on the precursor cholesterol (Woolley, 2007). Estrogens can be directly converted from cholesterol in the brain or from circulating androgens produced in the steriogenic organs outside the brain (Cornil et al., 2006; Srivastava et al., 2013). It is aromatase cytochrome P450 (aromatase) however, that is the final enzyme (Simpson et al., 1994) and the rate-limiting step (Srivastava et al., 2013) of the biosynthesis of estradiol producing three estrogen compounds: estrone, estriol and estradiol, of which estradiol is the most potent. Estradiol levels are higher in the hippocampus, picomolar to nanomolars, compared to circulating levels, lower picomolars in both male and female rats (Hojo et al., 2009). Hippocampal estradiol levels are also higher in OVX female rats compared to the circulating estradiol levels during all stages of the estrous cycle (Kato et al., 2013). This is thought to be a direct consequence of de novo synthesised estrogens. Critically, only high concentrations of estrogens elicit rapid effects (Srivastava et al., 2013). Therefore, circulating levels may not be
high enough to induce any rapid effects within the brain, and thus, it is likely that locally produced estradiol exert these effects (Cornil et al., 2006; Hojo et al., 2009). Additionally, estradiol is synthesised de novo in both male (Hojo et al., 2004) and female (Prange-Kiel et al., 2003) hippocampus suggesting that this is not unique to females. It is also important to consider how circulating estrogens show no specificity within the brain, as changes in estradiol levels would impact all areas of the brain expressing ERs equally. Conversely, locally produced estrogens could regulate cellular functions in a spatially restricted manner (Cornil et al., 2006; Srivastava et al., 2013). For estradiol to regulate rapid effects in the brain, it would need to be synthesised locally and thus, aromatase would have to be locally available. The following sections describe the localisation of aromatase within the brain and discuss its effects on synaptic plasticity.

LOCALISATION OF AROMATASE

Aromatase has been localised to a number of brain regions in rodents including the amygdala, cortex, hippocampus and the hypothalamus (MacLusky et al., 1994). In humans, aromatase expression has also been demonstrated in the male and female cortex (Yague et al., 2006). Although found in glial cells, this enzyme is highly expressed in pyramidal cells (Yague et al., 2006). Aromatase is also present at synapses (Hojo et al., 2004; MacLusky et al., 1994; Srivastava et al., 2010; Yague et al., 2008). Specifically, aromatase localises pre-synaptically (Cornil et al., 2012; Naftolin et al., 1996; Remage-Healey et al., 2011; Srivastava et al., 2010); co-localisation with pre-synaptic marker bassoon, and axonal marker tau5 was previously observed in the cortex in vitro (Srivastava et al., 2010). This study also reported co-localisation with post-synaptic density protein 95 (PSD-95), an abundant protein found at PSDs. Other studies have also described a post-synaptic localisation (Naftolin et al., 1996; Prange-Kiel et al., 2006). Thus, aromatase is ideally placed to drive the synthesis of estradiol both pre-/post-synaptically to regulate synaptic behaviour (Srivastava et al., 2013).
ROLE IN PLASTICITY

The role of hippocampally synthesised estradiol in synaptic plasticity has been widely examined. Aromatase inhibition, via letrozole, decreases estradiol levels (Prange-Kiel et al., 2003) and also orchestrates the decrease in dendritic spines, synaptic protein levels of pre-synaptic synaptophysin and post-synaptic spinophilin and number of pre-synaptic boutons (Kretz et al., 2004; Prange-Kiel et al., 2006; Vierk et al., 2012; Zhou et al., 2010). Physiologically, letrozole impairs long term potentiation (LTP) in male and female rats (Vierk et al., 2012), a form of synaptic plasticity thought to underlie memory formation (Takeuchi et al., 2014). Behaviourally, letrozole impairs object recognition (OR) and object placement (OP) memory in OVX female mice (Tuscher et al., 2016a). Thus, hippocampally synthesised estradiol has been shown to contribute to mediate hippocampal structural plasticity, physiology and behaviour.

**Estrogen receptors in the hippocampus**

ERs AND THEIR LOCALISATION

Local synthesis of estradiol and its ensuing local actions on structural plasticity suggest receptors specific for estradiol would also be appropriately localised. Estrogen receptors (ERs) alpha (ERα) and beta (ERβ), and the more recently identified G-protein coupled estrogen receptor 1 (GPER1; formally GPR30) have been implicated in mediating the rapid estrogen signalling in both cortex and hippocampus (Hughes et al., 2009; McEwen et al., 2001; Romano and Gorelick, 2018; Srivastava and Evans, 2013) and have received the most attention in research. It should be noted that there are other novel identified estrogen binding proteins that may be mediating estradiol’s effects such as ER-X or Gq protein-coupled membrane-initiated estrogen receptor (Gq-mER) (Micevych and Dominguez, 2009).

Classical, or nuclear, ERα and ERβ are structurally related and derive from the class I nuclear receptor superfamily acting as ligand-activated transcription factors (Hughes et al., 2009; Rainville et al., 2015). They are composed of
several functional domains: A/B or NH₂-terminal domain; C or DNA-binding domain; and the D/E/F or ligand binding domains (Nilsson et al., 2001). Upon ligand binding, the nuclear ERs undergo conformational changes followed by dimerization, their subsequent translocation from the cytoplasm to the nucleus where they bind to estrogen response elements (ERE) in the promoters of genes thereby, mediating gene transcription (Hughes et al., 2009). In contrast, GPER1 is a seven-transmembrane G-protein coupled receptor (Waters et al., 2015) and it has been suggested that its interaction with PSD-95 allows it to reside at the cell membrane (Akama et al., 2013). Interestingly, ERα and ERβ have also been found localized to the cell membrane (Micevych and Dominguez, 2009) and it is the membrane localized ERα and ERβ that are thought to contribute to the rapid effects of estradiol (Micevych and Mermelstein, 2008), which is discussed in the next sub-section. Razandi et al. (1999) demonstrated that membrane bound ERα and ERβ are derived from the same gene as nuclear ERα and ERβ in Chinese hamster ovary (CHO) cells. This suggests that the same nuclear-localised protein is capable of being targeted to the membrane. The mechanisms by which ERs are trafficked to cell membranes are not well understood nevertheless, two mechanisms have been thought to contribute to this (Almey et al., 2015). Studies have suggested post-translation modifications such as palmitoylation, which is known to promote membrane localization of proteins (Levin, 2010), play a pivotal role. Palmitoylation of cysteine sites in the ligand binding (E) domain of the ERs have been shown to be necessary for membrane localization in human cancer cells (HeLa) (Acconcia et al., 2005) and CHO cells (Razandi et al., 2003). Other studies have shown that ERs interact with scaffolding proteins, caveolins, that are found within caveolae within the plasma membrane (Almey et al., 2015). In support, knocking down caveolin was found to decrease the expression of membrane ERα in the hypothalamus (Christensen and Micevych, 2012). It has been suggested that palmitoylation of ERα is what allows the association with caveolin and, its subsequent translocation to cell membranes (Pedram et al., 2007). Although other mechanisms have been proposed, these two mechanisms have convincingly demonstrated how nuclear ERs may translocate to the cell membranes (Almey et al., 2015; Levin, 2010). A caveat of these studies is that the majority have focussed on ERα than both ERs and consequently, more research is warranted to confirm whether these
mechanisms dictate the trafficking of both ERs and if any other mechanisms are also necessary.

ERα and ERβ have differential distribution in the brain between region, sexes and age between species (Hughes et al., 2009; Sellers et al., 2015a). ERα mRNA and protein expression have both been predominantly found in the hypothalamus and amygdala whereas, ERβ predominantly found in the hippocampus formation, cerebral cortex and thalamus (Gonzalez et al., 2007; Hughes et al., 2009; Mitra et al., 2003); their putative locations suggest they have distinct roles in the brain. GPER1 is also expressed highly in the cortex and hippocampus (Almey et al., 2015; Srivastava and Evans, 2013). All three have also been localised in glial cells suggesting estradiol also mediates non-neuronal actions in the brain (Chaban et al., 2004; Waters et al., 2015). Critically, studies have denoted the presence of ERα, ERβ and GPER1 at synapses and other extra-nuclear locations such as, axon terminals within the hippocampus and cortex (Almey et al., 2015; Milner et al., 2001, 2005; Sellers et al., 2015b; Waters et al., 2015). Additionally, all three receptors have also been localised to interneurons within the rodent hippocampus (Milner et al., 2001, 2005; Waters et al., 2015). Thus, their localisation is ideal to rapidly regulate synaptic plasticity.

ER SIGNALLING

Traditionally, estrogen signalling was thought to be mediated by estrogens binding to classical ERα and ERβ, where the translocation to the nucleus result in the classical ‘genomic’ effects (Frick, 2015; Galea et al., 2017; Srivastava et al., 2013). These would take hours to days to manifest. Alternatively, the rapid effects or the ‘non-genomic’ effects are thought to be mediated by membrane, or extra-nuclear, ERs (Luine and Frankfurt, 2012; Srivastava et al., 2013). Employing specific ER agonists, investigations have uncovered specialised roles for the ERs within the hippocampus. Activation of specific ERs has been found to enhance synaptic plasticity within the hippocampus, and the cortex and improve hippocampal-dependent learning and memory (Choleris et al., 2012; Frick, 2015; Luine and Frankfurt, 2012; Sellers et al., 2015a; Srivastava et al., 2013), which will be explored in the following sections. There is accumulating
evidence suggesting that estradiol can initiate membrane-signalling pathways via ERs within 5-10 minutes *in vitro* and *in vivo* in the cortex and hippocampus (Frick, 2015; Luine and Frankfurt, 2012; Sellers et al., 2015a). The activation of multiple kinase pathways is thought to be critical in mediating estradiol-mediated enhancement of memory within the hippocampus (Frick and Kim, 2018), which are discussed in greater detail later. However, it is also appreciated that ERα and ERβ can interact with other receptors such as group I metabotropic glutamate receptor (mGluR) and group II mGluR within female-derived hippocampal neurons (Boulware et al., 2005). This study reported that whilst only ERα was found to activate group I mGluR signalling, both ERα and ERβ were found to activate group II mGluR signalling. ERs are therefore, ideally localised to rapidly regulate synaptic changes and activate membrane-initiated signalling cascades, which may contribute to hippocampal synaptic plasticity. As a consequence, a number of ERs may mediate estradiol signalling by coupling to different intracellular signalling cascades, directly or via other receptors leading to the activation of specific signalling proteins (Sellers et al., 2015a). This could lead to a number of cellular effects driven either genomically through gene transcription or non-genomically such as, inducing local protein synthesis (Figure 1.1).
Rapid estrogen membrane-signalling. The rapid effects of estradiol are mediated by a number of different estrogen receptors (ERs) such as, ERα, ERβ and GPER1. ERs couple to intracellular signalling cascades potentially directly or through the activation of other receptors. This leads to the activation of a number of kinases such as the extracellular signal-regulated kinase 1/2 ERK1/2 (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) (P13/Akt) and mammalian target of rapamycin (mTOR). The subsequent activation can lead to the modulation of several cellular effects such as cytoskeletal reorganisation and trafficking of proteins. It can also lead to gene transcription or the regulation of local protein synthesis. Adapted from Sellers et al. (2015a).

MODULATION OF COGNITION AND UNDERLYING MECHANISMS

Hippocampal memory

One of the most studied effects of estradiol in the hippocampus is its beneficial effects on learning and memory (Frick, 2015; Luine and Frankfurt, 2012). There has been considerable evidence in OVX female rodents in comparison to the paucity of studies in male rodents. Nevertheless, estradiol-mediated
enhancement of memory is not unique to OVX females (Frick, 2015). A small number of studies have assessed memory in gonadally intact females largely because of the challenges faced between fluctuating hormone levels over the estrous cycle and the protocols for assessing memory that last over a few days (Tuscher et al., 2015). However, studies have illustrated enhanced performance on memory tasks during proestrus, where estradiol levels are high, compared to diestrus and estrus, when levels are lower in rats (Walf et al., 2006) and mice (Sánchez-Andrade and Kendrick, 2011). A myriad of studies has measured effects of estradiol on memory acquisition and consolidation mostly by employing OR, OP and social discrimination tasks, although other behavioural tasks have also been employed. These tasks comprise of two identical objects or conspecifics that rodents explore during the training period (T1). Following an inter-trial delay either: a new object is substituted for a new one (OR); one of the objects is moved to a different location (OP); or a conspecific is substituted for a novel one. Memory acquisition or consolidation is determined during the testing period (T2) measured by a greater exploration time with the novel/relocated object or novel animal (Ervin et al., 2015; Luine, 2014). Timing of estradiol administration and behavioural testing can categorise rapid or long-term effects of estradiol on memory (Paletta et al., 2018). Estradiol affects learning and memory via both ‘genomic’ and ‘non-genomic’ mechanisms and these mechanisms are not mutually exclusive (Sheppard et al., 2018). A number of studies that chronically applied estradiol days to weeks prior training found memory enhancements in a number of memory tasks in both gonadectomised (GDX) male (Luine and Rodriguez, 1994) and OVX females (Fonseca et al., 2013; Jacome et al., 2010; Luine et al., 1998; Vedder et al., 2013). The time-frame of these studies suggest that estradiol may utilise gene transcription to enhance memory. Historically, memory research was directed towards the genomic actions of estradiol however, the data pointing towards the rapid effects of estradiol in the hippocampus are plenteous.

ACUTE ESTRADIOL TREATMENT ON MEMORY – ACQUISITION STUDIES IN OVX FEMALE RODENTS

One of the first studies to illustrate rapid effects of estradiol on memory demonstrated that estradiol administered either 30 minutes before training or
immediately after enhanced OR and OP when tested 4 hours later in OVX female rats (Luine et al., 2003). This was recapitulated in a more rapid learning paradigm by administering estradiol 15 minutes before training, followed by testing after 25 minutes in OVX female mice (Phan et al., 2012); enhanced social recognition was also observed within the same time-frame. This study was a first of its kind to illustrate improved learning within 40 minutes of estradiol administration with the advantage of using a time-frame where memory is reported to be independent of transcription (Phan et al., 2012). Thus, suggesting that these rapid estrogenic actions on learning are possibly non-genomic.

ACUTE ESTRADIOL TREATMENT ON MEMORY – CONSOLIDATION STUDIES IN OVX FEMALES RODENTS

A number of studies from both Victoria Luine and Karyn Frick's groups have assessed estradiol's effects on memory consolidation by administering estradiol immediately after training, with varying inter-trial delays (Sheppard et al., 2018). Estradiol administration immediately after training, but not after a delay of 2 hours (Luine et al., 2003) or 45 minutes (Inagaki et al., 2010) enhanced both OR and OP in OVX female rats. Other studies have reported that estradiol enhanced OR and OP when it was administered immediately (Walf et al., 2008) but not after 1 hour (Walf et al., 2006) or 1.5 hours (Frye et al., 2007) respectively in OVX female rats. The testing occurred 4 hours after training for all the investigations. Furthermore, these findings have been mirrored in OVX female mice. Estradiol enhanced OR when administered immediately after training (Fan et al., 2010; Fortress et al., 2013; Gresack and Frick, 2006) but not after 3 hours (Fernandez et al., 2008) when tested 24-48 hours after training. Critically, some studies have found no such effect of estradiol enhancing hippocampal memory in OVX female rats using other memory tasks, such as the radial arm maze task (Galea et al., 2001). Collectively, these studies report that estradiol enhances memory consolidation only when administered after training but that a delay of 45 minutes or longer has no effect. This provides a specific time-frame as to when estradiol is having an effect.
MEMORY STUDIES IN MALE RODENTS

The rapid memory enhancing effects of estradiol has been investigated to a lesser extent in male rodents compared to females. A recent study has illustrated that estradiol administered post training enhanced OP in GDX male rats (Jacome et al., 2016). Other unpublished preliminary work from Karyn Frick’s laboratory suggests that both OR and OP are enhanced following estradiol administration after training in gonadally intact male mice (Koss and Frick, 2017). Other spatial memory tasks have shown estradiol-mediated enhancement in the Morris water maze task in gonadally intact male mice (Packard and Teather, 1997) and as previously mentioned, GDX male rats (Luine and Rodriguez, 1994). Critically, this enhancement was not observed in gonadally intact males more recently (Moradpour et al., 2006). Thus, a limited number of studies suggest estradiol can rapidly enhance memory consolidation in male rodents, but further research is warranted. Thus far, the rapid effects of estradiol has not been studied in the context of social discrimination however, many studies have looked the long-term effects of estradiol in a number of ER KO rodent models (Choleris et al., 2012). Although more research is warranted in the male hippocampus, estradiol seems to have a synonymous effect in enhancing memory as observed in OVX females.

Overall, these studies show that estradiol has no effect when administered 45 minutes after training, suggesting that estradiol is contributing to the memory consolidation quite rapidly. In support, paradigms where estradiol’s effects were observed after 40 minutes (Phan et al., 2012) occur in too rapid of a time-frame to be dependent on genomic effects (Paletta et al., 2018). Indeed, these enhancements are observed across different species, training protocols, routes of administration suggesting estradiol is reliably enhancing specific hippocampal memory (Frick and Kim, 2018). Overall, rodent studies have provided an insight into the rapid enhancing effects of estradiol in hippocampal memory consolidation; these studies are summarised in Figure 1.2. The underlying molecular mechanisms that mediate this rapid memory consolidation are however, unclear. More so, whether these mechanisms are synonymous between sexes is also unclear.
Figure 1.2 Estrogen rapidly enhances memory in rodents. Diagram adapted from Dr. Deepak Srivastava (personal communication) showing a summary of different studies where estradiol has been shown to enhance object recognition (OR), object placement (OP) and social recognition memories. Two identical objects or conspecifics are presented to rodents during the training period (T1). Following an inter-trial delay either: a new object is substituted for a new one (OR); one of the objects is moved to a different location (OP); or a conspecific is substituted for a novel one. Studies where estradiol was administered 15-30 minutes before, or immediately after training show an enhanced performance in OVX female rodents when tested 4-48 hours post-training. When administered after a delay post-training, estradiol has no enhancing effects on memory.

Molecular mechanisms underlying memory enhancement

ERs IMPLICATED IN ENHANCING HIPPOCAMPAL MEMORY

Multiple lines of evidence have tried to tackle the question of which ER is required in mediating these memory enhancements. Although this has been investigated using rodent ER-specific KO models and specific ER agonist, a lot of this largely remains unclear. Much of the work using the rapid learning paradigm have shown ERα, through agonist 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triy]trisphenol (PPT) (Gabor et al., 2015; Phan et al., 2012) and GPER1 agonist rel-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-
cyclopenta[c]quinolin-8-yl]-ethanone (G1) (Gabor et al., 2015) to mediate memory enhancing effects of estradiol in OR, OP and social discrimination. Whereas, other studies administering agonists, including ERβ agonists 7-Bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070; 070) and 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), and specific antagonists immediately following training have implicated both ERα and ERβ (Boulware et al., 2013; Kim and Frick, 2017; Pereira et al., 2014; Walf et al., 2006). ER-specific KO models have suggested a more prominent role for ERβ (Liu et al., 2008; Walf et al., 2008). Collectively, these results allude to the role of both ERα and ERβ in enhancing memory. Nevertheless, more studies are warranted to confirm this. Furthermore, studies are also required to explore the role of GPER1 and investigate which ER is important in males.

CELL SIGNALLING CASCADES REGULATING ESTRADIOL-INDUCED MEMORY CONSOLIDATION

It can be reasoned that if estradiol is differentially employing ERs to mediate different types of memory, that different signalling pathways may also be involved in mediating different types of memory. Moreover, these pathways may not be synonymous in both sexes. Estradiol has been demonstrated to rapidly activate kinases that have been implicated in memory enhancement such as the extracellular signal-regulated kinase 1/2 ERK1/2 (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) (P13/Akt) and mammalian target of rapamycin (mTOR) within 5 minutes in vivo (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013), in vitro (Briz and Baudry, 2014; Sarkar et al., 2010; Sellers et al., 2015b) and in various cell lines (Akama and McEwen, 2003; Sarkar et al., 2010). Activation of ERK signalling has been implicated in the consolidation of spatial (Blum et al., 1999) and recognition memories (Kelly et al., 2003).

Majority of the studies have investigated the role of these kinases in the memory enhancing effects in the hippocampus and have implicated both ERK and P13/Akt pathways in OVX female rodents. ERK phosphorylation inhibitor, U0126, has been shown to prevent estradiol-mediated increase in OR (Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010) and OP (Kim et
al., 2016) when administered immediately after training in mice. Preliminary findings from Elena Choleris' laboratory have demonstrated that ERK activation inhibition 15 minutes prior to training is sufficient to impair social recognition in mice (Sheppard et al., 2016). Thus, these results suggest that ERK activation is critical for estradiol's effects on both acquisition and consolidation of memories. Additionally, the PI3K/Akt pathway has demonstrated to be critical in LTP and recognition memory (Horwood et al., 2006). Inhibiting the PI3K/Akt pathway has been shown to block the estradiol-mediated phosphorylation of ERK and memory enhancement (Fan et al., 2010; Fortress et al., 2013; Lewis et al., 2008). Interestingly, Elena Choleris’ laboratory has demonstrated that LY294002, a PI3K inhibitor, administration 15 minutes prior to training blocks estradiol from enhancing social recognition (Sheppard et al., 2017). Thus, both ERK and PI3K/Akt signalling pathways are necessary for estradiol to promote memory acquisition and consolidation. Additionally, both ERK and PI3K signalling pathways activate mTOR and it is posited that mTOR may be the convergence point for both (Richter and Klann, 2009). mTOR was first demonstrated to be critical in contributing in NMDAR-dependent late-phase of LTP (L-LTP) (Tang and Schuman, 2002) since then, its role in synaptic plasticity is greatly appreciated specifically, it's role in mediating local protein synthesis (Hoeffer and Klann, 2010) and memory enhancement (Bekinschtein et al., 2007). Fortress et al. (2013) additionally found that estradiol-mediated enhancement of OR was blocked in the presence of mTOR inhibitor, rapamycin. This suggests that all these pathways work together to mediate memory enhancing effects of estradiol however, whether they work in parallel, or independently is currently unknown. However, this study postulated that estradiol may be engaging protein synthesis within spines to enhance memory consolidation in rodents. This remains to be tested, but there is evidence that estradiol engages protein synthesis machinery.

Remodelling of synaptic structure and function

The studies reported above detailing estradiol-mediated rapid memory enhancements suggest that estradiol may be remodelling synaptic structure and function within the same time-frame. Indeed, classic studies by Catherine
Woolley, Elizabeth Gould and Bruce McEwen demonstrating spine and synaptic remodelling in response to the estrous cycle and estradiol treatment paved the way for future studies to investigate this. Following this, a number of studies have illustrated that chronic estradiol treatment enhances dendritic spine density (Luine and Frankfurt, 2012). More recently, a number of laboratories including ours, have reported that estradiol rapidly promotes spinogenesis in vitro (Sellers et al., 2015b; Srivastava et al., 2008, 2010) and in vivo (Inagaki et al., 2012; MacLusky et al., 2005; Phan et al., 2011, 2012, 2015; Tuscher et al., 2016b), within 30 minutes in both cortex and hippocampus. This increase is seen as early as 15 minutes in vitro but this was transient as spinogenesis returned back to basal levels after 45 to 60 minutes (Srivastava et al., 2008). Additionally, Srivastava et al. (2008) reported the internalisation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA1, and subsequent insertion of NMDAR subunit GluN1 causing the presence of silent synapses, was found to accompany spinogenesis. The spine increase was transient but was sustained by subsequent NMDAR activation, which led to the two-step wiring plasticity model (Srivastava et al., 2008). The model suggests that the increase in spines and generation of silent synapses places the spines in a 'primed' state, ready to respond to stimuli with a greater efficacy (Sellers et al., 2015a). In the absence of a stimulus, the novel spines are eliminated. However, upon a stimulus, these novel spines are sustained causing an increase in synaptic connectivity and transmission. Thus, priming estradiol-induced spines may contribute to the estradiol-mediated memory enhancements seen within 40 minutes (Sellers et al., 2015b). Indeed, enhanced performance in memory tasks was reported to be accompanied by increased spinogenesis within 40 minutes estradiol treatment (Phan et al., 2012). Therefore, suggesting a potential role for spinogenesis contributing to the estradiol-mediated acquisition of new memories. Interestingly, this initial increase in spine density at 30 minutes has been demonstrated to be independent of protein translation (Srivastava et al., 2008) but these spines encompass the molecular machinery needed to be functional (Sellers et al., 2015b). In contrast to what is seen in the cortex in vitro, estradiol-mediated spinogenesis is still observed after 2 hours in the hippocampus in vivo (Mukai et al., 2007; Murakami et al., 2015). Consistent with this, estradiol enhances LTP in the hippocampus within 2 hours (Liu et al., 2008). In vitro, ERβ has been
implicated in increasing spines (Srivastava et al., 2010) within the cortex whereas, *in vivo* it is ERα that has been implicated in the hippocampus (Mukai et al., 2007; Murakami et al., 2015; Phan et al., 2011). Thus, different ERs may be important in mediating spine changes within the cortex and hippocampus thereby, suggesting that different signalling pathways may be important in mediating these effects. Studies from our laboratory have shown that both ERK and PI3K/Akt, but not mTOR, are needed for estradiol-mediated increase in spines after 30 minutes within the cortex whereas, mTOR and ERK are both necessary for estradiol to increase after 2 hours in the hippocampus (Tuscher et al., 2016b). Given that mTOR has been implicated in local protein synthesis, estrogenic activation of mTOR-mediated protein synthesis may play a role in supporting the maintenance of new spines.

**A role for local protein synthesis**

The study by Fortress et al. (2013) was the first study to highlight a role for mTOR-dependent protein synthesis to contribute to the memory enhancing effects of estradiol. Particularly, they found that estradiol activated mTOR, through ERK and PI3/Akt, which led to an increase in the phosphorylation of both p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) within 5 min in dorsolateral hippocampal slices, both of which are implicated in regulating local protein synthesis (Costa-Mattioli et al., 2009; Hoeffer and Klann, 2010). Estradiol’s rapid activation of local protein synthesis machinery has been reported multiple times both *in vitro* (Akama and McEwen, 2003; Sarkar et al., 2010) and *in vivo* (Fortress et al., 2013). Akama and McEwen (2003) have previously reported increased new protein synthesis of PSD-95 along dendrites of a differentiated hormone-responsive neuroblastoma cell line, NG108-15 (d-NG108-15) after 6 hour estradiol treatment. However, Sarkar et al. (2010) reported increased mRNA translation of a GFP expression based on a CAMKIIα 3’ untranslated region (UTR) reporter in dendrites over 1 hour in primary neurons. These studies suggest that estradiol could be engaging local protein synthesis machinery both *in vivo* and *in vitro* to mediate increased dendritic translation over 1 hour and that PSD-95 could be a potential
target to investigate. To date, estradiol signalling has not been directly linked to protein synthesis although, some studies suggest this may be the case.

A consideration for sex differences

Multiple lines of evidence presented in the previous sections have outlined the molecular mechanisms that contribute to the effect of estradiol on structural plasticity and enhancing memory. However, there is a gap in the literature when it concerns what is known about which signalling pathways or ERs are involved in mediating structural plasticity and enhancing memory in males. Specifically, Oberlander and Woolley (2016) have shown that estradiol utilises two different receptors in males and OVX females to modulate the same synaptic plasticity. In support, biological differences between males and females are found at multiple levels, and within the hippocampus (Choleris et al., 2018), which could have differential implications in cognition and behaviour. Thus, there is a need for more studies to consider both sexes when assessing estradiol’s effects on neuronal transmission, structural plasticity or memory. In doing so, our knowledge of estradiol’s effects in the male and female brain separately will be greater.

LOCAL PROTEIN SYNTHESIS

Local vs. global protein synthesis

Synaptic type and strength is dependent on the synaptic proteome and thus, the regulation of the proteins that inhabit synapses can drive synaptic plasticity and general neuronal homeostasis (Hafner et al., 2018). This can be achieved through the degradation of proteins or the synthesis of new proteins. Protein synthesis allows the replenishment of the proteome in subcellular compartments within the neuronal cell in for example, at synapses. Recently, the complexity of the synaptic proteome was elegantly demonstrated by Dörrbaum et al., (2018) by illustrating the half-life of proteins present at both excitatory and inhibitory synapses in the hippocampus in vitro (Figure 1.3). A number of proteins at the PSD demonstrated short half-lives, compared to those
at the excitatory pre-synaptic or inhibitory post-synaptic compartments. This highlights the dynamic changes that PSD would endure and the need for protein replenishment. New protein synthesis is thought to control dendritic processes, such as synaptic plasticity, that have been thought to underlie or contribute to memory (Sutton and Schuman, 2006). Others have shown protein synthesis contributes to other neuronal processes such as axonal growth cone navigation and neuronal differentiation and has also been shown to occur in non-neuronal cells such as astrocytes (Vlatkovic and Schuman, 2016). However, why protein synthesis is so important in many neuronal and specifically, synaptic processes is still not fully understood.

Figure 1.3 The complexity of the synaptic proteome. A number of proteins have been identified to be localised to and important for the functioning of glutamatergic (A) or glycinergic/GABAergic (B) synapses. The half-lives of these proteins are presented, and colour coded by red representing more short-lived and green representing more long-lived proteins (C). This highlights the
dynamic changes that occur to the synaptic proteome. From Dörrbaum et al., (2018).

The process of protein synthesis follows the transcription of messenger RNA (mRNA) and involves the initiation, elongation and termination of polypeptide chains of amino acids (Sonenberg and Hinnebusch, 2009). The dogma that protein synthesis was restricted to the cell soma had been challenged and a plethora of studies have followed since polyribosomes, the machinery that drives the translation of mRNAs, were discovered in dendrites (Steward and Levy, 1982). The ability for neurons to locally control proteome remodelling circumvents the communication required between the nucleus and synapse to organise the synthesis and transportation of proteins, which can take from hours to days as dendrites are remote from the cell body (Rangaraju et al., 2017). Thus, local protein synthesis can establish and maintain proteins at synapses independently. A number of studies have demonstrated local protein synthesis to occur along dendrites (Pfeiffer and Huber, 2006; Steward and Schuman, 2001) and fewer have also shown this in axons (Lin and Holt, 2007) and astrocytes (e.g. Sakers et al., 2017). A number of studies by Christine Holt, and others, have implicated axonal local protein synthesis in the regulation of growth cone navigation; one such illustrating this retinal axons of the *Xenopus* (Campbell and Holt, 2001). A later study by Leung et al. (2006) identified β-actin as one of the proteins that is translated and contributes to this. Indeed, if protein synthesis is occurring in distal parts of the neuron, the presence of the appropriate machinery and substrate are necessary.

Protein synthesis machinery at dendrites & axons

DENDRITES

Succeeding the earlier findings of Steward and Levy (1982), other machinery necessary for protein translation initiation was also discovered to be localised to dendritic compartments. These included transfer RNA (tRNA) and aminoacyl-tRNA synthetases, which both aid in the transport of mRNA to the ribosome, in primary hippocampal neurons (Tiedge and Brosius, 1996). They also reported
the presence of initiation and elongation factors such as eukaryotic initiation factor 2 (eIF2) and eukaryotic elongation factor 2 (eEF2) along dendrites. Others have reported the presence of other elongation factors such as eukaryotic translation initiation factor 4E (eIF4E) (Tang et al., 2002). In addition to the machinery, the substrates to initiate protein translation have also been identified at dendrites. Bagni et al., (2000) found that specific mRNA transcripts for calcium/calmodulin-dependent kinase II α subunit (CAMKIIα), Arc and InsP3R1 mRNA were all associated with polyribosomes in cortical synaptosome preparations. Since, studies have demonstrated large pools of dendritically localised mRNA (Poon et al., 2006; Zhong et al., 2006). More recently, approximately 2,550 mRNA were identified to be dendritically localised through deep RNA sequencing in both primary hippocampal neurons and hippocampal sections (Cajigas et al., 2012) (Figure 1.4). Among which were transcripts encoding proteins involved in synaptic plasticity such as PSD-95, GluA1, Homer 1, and Shank 1. Interestingly, the mRNA encoding for proteins that regulate protein translation initiation and elongation such as eIF4E and eEF2, and RNA binding proteins such as fragile X mental retardation protein (FMRP) were also identified. Thus, not only proteins implicated in synaptic proteins can be replenished locally, but also those that regulate protein translation. Evidence that local translation occurs was provided by Rao and Steward (1991) at synapses using synaptosome preparations. Following this, Torre and Steward (1992) demonstrated that protein synthesis specifically occurred at dendrites. Thus, the presence of translation machinery and a number of transcripts highlights the importance of local protein synthesis in a number of neuronal, specifically synaptic, function.

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2 Human inositol 1,4,5-trisphosphate type-1 receptor
Figure 1.4 The population of mRNA transcripts found in synaptic compartments. A schematic from Cajigas et al., (2012) highlighting the mRNA transcripts coding proteins important in synaptic function and protein translation found in the synaptic neuropil revealed by deep sequencing in CA1 hippocampus.

AXONS

The wealth of data implicating protein translation machinery is greater in dendrites than axons. Nevertheless, evidence from squid giant axons demonstrated that mRNA, ribosomal RNAs (rRNAs) and actively translating polyribosomes are all present (Jung et al., 2012). Ribosomes have also been localised in mammalian mature axons (Koenig et al., 2000) and polyribosomes have been found in growth cones (Bassell et al., 1998). This suggests that axons may encompass the machinery to coordinate protein synthesis. In support, a recent study performed a deep sequencing analysis of ribosome-bound mRNA in developing and mature retinal axons and found transcriptomes involved in synapse function such as glutamate receptors, SNARE proteins, axonal survival and guidance (Shigeoka et al., 2016). Thus, both the machinery and substrate needed for translation can be found within the axonal terminal. Interestingly, Shigeoka et al.’s (2016) data showed that mature axons translate
mRNA for synaptic transmission and thus, pre-synaptic local protein synthesis could contribute to post-synaptic maintenance.

**Role in synaptic plasticity and memory**

Local protein synthesis at dendrites is deemed a critical component of long-lasting changes in synaptic plasticity (Klann et al., 2004). Two forms of synaptic plasticity, LTP and long-term depression (LTD) consist of two phases, an early phase that is independent of protein synthesis and a more persistent late phase dependent on protein synthesis (Sutton and Schuman, 2006). Thus, new proteins are required for long lasting changes. Early studies using protein synthesis inhibitors illustrated a decay in the early phase of LTP (E-LTP) *in vitro* (Stanton and Sarvey, 1984) and *in vivo* (Frey et al., 1988) upon administration. However, it was thought that new proteins were a result of newly transcribed mRNA (Pfeiffer and Huber, 2006). It was a series of experiments by Kang and Schuman (1995, 1996) who provided evidence that it was local protein synthesis that was critical for synaptic enhancement. Upon the application of protein synthesis inhibitors, anisomycin and cyclohexamide, the increase in synaptic potentiation induced by brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were blocked in CA1 dendrites severed from the cell body. This suggested that protein synthesis in distal parts of the cell could contribute to synaptic plasticity. Another study from Erin Schuman’s laboratory showed BDNF-induced protein synthesis of a green fluorescent protein (GFP) reporter flanked by the 5’ and 3’ UTRs of CAMKIIα in severed dendrites *in vitro* (Aakalu et al., 2001). Thus, providing a direct visualisation of new CAMKIIα protein synthesis within dendrites. Consistent with this, Bradshaw et al. (2003) locally applied protein synthesis inhibitors to CA1 dendrites during LTP induction subsequently blocking L-LTP. In support, Vickers et al. (2005) found that L-LTP could be still be induced in dendrites isolated from the soma and subsequently blocked with protein synthesis inhibitors in hippocampal slices. mGluR activation also facilitates LTP persistence, which has been shown to be dependent on protein, but not mRNA, synthesis (Raymond et al., 2000). mGluR activation also contributes to a form of LTD (mGluR-LTD). Huber et al. (2000) demonstrated that mGluR agonist, (RS)-3,5-Dihydroxyphenylglycine (DHPG),
application on severed dendrites of CA1 induced LTD, which was blocked in the presence of protein synthesis inhibitors. Collectively, these studies exhibit the critical role of local protein synthesis in modulating synaptic plasticity. LTP is thought to underlie memory formation (Takeuchi et al., 2014) and so it can be reasoned that local protein synthesis could contribute to different types of memory. Very few have directly measured the necessity for local protein synthesis in memory enhancement. Nevertheless, Miller et al., (2002) employed a CAMKI\(\alpha\) mRNA deficient mouse to test whether this would consequently disrupt memory; the dendritic localisation was interfered by mutating its 3’ UTR. This was upon the notion that CAMKI\(\alpha\) has been considered necessary for LTP (Lisman et al., 2002). Compared to their wild type (WT) counterparts, the performance of the CAMKI\(\alpha\) deficient mice was impaired in the hippocampal dependent tasks, Morris water maze and OR and the amygdala dependent task, contextual fear conditioning. Thus, suggesting new CAMKI\(\alpha\) synthesis is critical in mediating these specific memories.

Regulation of local protein synthesis

SIGNALLING PATHWAYS

Two signalling pathways that have received a lot of attention in contributing to the regulation of protein synthesis are the mTOR and ERK signalling pathways. These pathways activate several downstream signalling molecules that play a role in protein translation initiation and regulation. Studies have also shown that ERK can activate mTOR (Winter et al., 2011) and also regulate translation machinery via mTOR (Roux et al., 2007; Tsokas et al., 2007; Winter et al., 2011). Thus, it is not clear whether these pathways act in parallel, or sequentially to regulate protein synthesis machinery. However, mTOR and ERK can both regulate various aspects of translation initiation.

The mTOR pathway stimulates local protein synthesis by phosphorylating key downstream translational targets including S6K1/2 and 4EBP1 (Costa-Mattioli et al., 2009; Hoeffer and Klann, 2010; Lipton and Sahin, 2014). Each of these regulates protein synthesis through different mechanisms. Activation of S6K1/2
promotes the phosphorylation of ribosomal protein S6 (RPS6), a member of the small 40S ribosomal complex. Its phosphorylation has been implicated in having a regulatory role in translation initiation (Thomas et al., 1982), required for L-LTP (Panja et al., 2009; Tsokas et al., 2005), and has recently been detected near active synapses (Pirbhoy et al., 2017). Interestingly, this activation near active synapses was demonstrated to be MAPK/ERK dependent. Thus, suggesting a point of convergence for both mTOR and ERK signalling pathways.

mTOR phosphorylation can additionally hyperphosphorylate 4E-BP1, which upon activation releases the translation repression on the cap binding protein eIF4E (Klann et al., 2004). 4EBP1 is typically bound to eIF4E and once 4EBP1 is phosphorylated, eIF4E is free to complex with eIF4G and other initiation proteins. Within the complex, eIF4E then directly binds with ‘the cap’ structure found on mRNAs - a methylated guanosine repeat at the 5’ UTR on the majority of eukaryotic mRNAs (Lipton and Sahin, 2014). eIF4E subsequently helps mRNAs to be recruited to ribosomes (Gingras et al., 1999). Phosphorylation of eIF4E has been demonstrated to increase translation of a subset of mRNAs that play a role in memory formation (Bramham et al., 2016). eIF4E has previously been demonstrated to co-localise with post-synaptic markers (Tang and Schuman, 2002) and translocate to dendritic spines following BDNF stimulation (Smart et al., 2003). BDNF stimulation has shown eIF4E to be phosphorylated in a mTOR-4EBP1 dependent manner in isolated dendrites (Takei et al., 2004) highlighting the importance of mTOR-dependent translation activation in local protein synthesis. Indeed, studies using mTOR inhibitor, rapamycin, have shown impairments in L-LTP (Tang and Schuman, 2002), BDNF-induced stimulation (Tang et al., 2002) and mGluR-LTD (Huber et al., 2001) implicating a critical role for mTOR in synaptic plasticity. Critically, BDNF stimulation also phosphorylates eIF4E in an ERK-dependent manner (Kelleher et al., 2004). Indeed, eIF4E itself can be phosphorylated by ERKs downstream targets MNK1/2 kinases at Serine 209 (Ser\textsuperscript{209}) (Banko et al., 2004; Waskiewicz et al., 1999). Thereby, suggesting an additional convergence for both mTOR and ERK signalling pathways. Interestingly, recently it has been shown that phosphorylation deficits in eIF4E triggers depression like phenotypes in male and female rodents (Aguilar-Valles et al., 2018).
DENDRITIC MICRORNAS

MiRNAs are small non-coding RNA molecules that act as post-transcriptional regulators of gene transcription (Bartel, 2009). miRNAs bind to the 3’UTR of the target mRNA and induces the reduction or repression of translation of the transcript (Schratt et al., 2006), a process recently found to be important in regulating protein synthesis at dendrites (Rajgor et al., 2018). MiRNA biogenesis involves the cleavage of the primary mRNA (pri-mRNA) in the nucleus by an enzyme complex including the nuclear ribonuclease III (RNaseIII) enzyme, Drosha, and a double-stranded RNA-binding domain protein, Pasha, to yield the precursor mRNA (pre-mRNA) (Kosik, 2006). The pre-mRNA is then transported to the cytoplasm and cleaved by RNaseIII enzyme, Dicer, resulting in mature miRNA (Kosik, 2006). MiRNAs associate with argonaute (Ago) proteins to form the RNA-induced silencing complex (RISC), which is then guided to the target mRNA for repression (Kosik, 2006).

Neuronal miRNAs have been demonstrated to be involved in neuronal development, spinogenesis and synaptic function and plenteous have been reported to be enriched in dendrites and at synapses (Schratt, 2009). They have also been localised to axons (Sasaki et al., 2014). Regulating miRNA activity contributes to controlling the synthesis of specific proteins that in turn regulate dendritic spine morphology, function, maintenance and thereby, synaptic plasticity. For example, Schratt et al. (2006) reported miR-134 localised at dendrites in primary rat hippocampal neurons and found it to repress the expression of synaptic protein Limk1, which regulates the actin cytoskeleton thereby, negatively controlling spine maturation; some miRNAs also positively regulate spine maturation such as miR-125a that targets PSD-95 mRNA (Rajgor and Hanley, 2016). Recently, Rajgor et al. (2018) proposed a mechanism for this; Limk1 translation is occluded at dendrites by miR-134 via the phosphorylation of Ago2 specifically through the Akt signalling pathway in response to NMDA stimulation which, resulted in spine shrinkage. Many other miRNAs have since been dendritically localised and associated with regulating mRNA translation of synaptic proteins (Rajgor and Hanley, 2016; Rajman and Schratt, 2017). However, how this process is regulated to control synaptic plasticity is unknown.
Evidence posits that miRNAs regulate gene expression specifically at the synapse (Schratt, 2009); the machinery to process miRNAs such as Dicer (Lugli et al., 2005) and pre-miRNAs (Bicker et al., 2013) have also been illustrated to localise near synapses suggesting that miRNAs could be generated locally near synapses. Sambandan et al. (2017) elegantly illustrated the generation miR-181a directly in dendritic spines by stimulating individual synapses via uncaging glutamate. What is more, a decrease in newly translated CAMKIIα, a target of miR-181a, was reported near the regions of pre-miR-181a. Sambandan et al. (2017) also document that pre-miRNA processing can by stimulated by neural activity specifically at individual synapses initiating a local decrease in protein synthesis of their target protein at that very synapse. Thereby, miRNAs can be generated quite rapidly and can contribute to the silencing of synaptic proteins during synaptic plasticity. In support, Hu et al. (2014) reported the upregulation of miR135 and downregulation of miR191 and therefore, protein translation were required for persistent changes in spine remodelling in LTD. There is still the need to understand how miRNAs orchestrate local mRNA translation in response to synaptic activity and whether it utilises similar mechanisms to that reported by Sambandan et al. (2017). Nevertheless, miRNAs play an important role in regulating protein synthesis to regulate various forms of synaptic plasticity.

CONTRIBUTION OF PRE-SYNAPTIC STRUCTURES

There is sufficient evidence for the occurrence of protein synthesis at post-synaptic regions of neurons in mediating changes in synaptic plasticity. However, very little is known about any contribution of the pre-synaptic structures. Interestingly, a first of its kind study by Hafner et al. (2018) have illustrated rapid translation occurring in both pre- and post-synaptic compartments within the hippocampus in vitro. They report that >100 mRNAs and an abundance of ribosomes were found to be localised within excitatory and inhibitory pre-synaptic compartments moreover, these compartments were found to be actively translating proteins in addition to the excitatory post-synaptic compartment. Critically, different forms of plasticity exhibited differential patterns of pre- and post-synaptic translation. Thus, this is the first
study to suggest that both pre- and post-synaptic compartments contribute to different types of synaptic plasticity and both should be considered when investigating the underlying molecular mechanisms.

**AIMS AND HYPOTHESES**

The literature presented here demonstrates that estradiol can rapidly modulate hippocampal memory in both male and female rodents. Studies have presented ERs and signalling pathways that may implicate this. Indeed, estradiol has been illustrated to reorganise the neural circuitry within the same time-frame. But the underlying molecular mechanisms of this enhancement are not fully understood. Corroborating the findings that demonstrate estradiol’s rapid actions on activating protein translation initiation machinery, and studies that show rapid dendritic translation, it can be reasoned that estradiol may rapidly increase protein synthesis, and this could contribute to its memory enhancing effects.

The following chapters will investigate the role of acute estrogen signalling in regulating local protein synthesis within the hippocampus. The hypothesis of this project therefore, is that **estradiol will increase protein synthesis in a local protein translation dependent manner**. Acute hippocampal slices from both male and OVX female mice and primary hippocampal neurons will be used in this study as majority of the literature implicates profound effects of estradiol in the hippocampus. Moreover, in light of the previous studies presented, there is a huge gap in the literature investigating the role of estradiol in the male hippocampus for example, the signalling pathways and ERs implicated in regulating estradiol-mediated memory are unknown. Thus, it was important to consider both sexes within this current investigation, as memory enhancing effects of estradiol can be observed in both sexes. The aims of this investigation therefore, are to:

1. Establish whether estradiol increases protein synthesis in the male and OVX female hippocampus;
2. Determine if both sexes use the same signalling pathway;
3. Identify which proteins may be locally translated;
4. Investigate which subcellular compartment the newly synthesised proteins are targeted to following estradiol treatment.
CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Mixed sex cortical and hippocampal cultures were prepared from Sprague-Dawley rat E18 embryos as previously described (Srivastava et al., 2008). The rats were obtained from Charles River Laboratories, United Kingdom, and habituated for 3 days before experimental procedures were carried out in accordance with the Home Office Animals (Scientific procedures) Act, United Kingdom, 1986.

All acute cortical and hippocampal slices were prepared from C57BL/6J mice between the ages of 10-12 weeks obtained from The Jackson Laboratory, Maine, United States. Both intact male and OVX female mice were used in the experimental procedures. The female mice were ovariectomised by the vendor at 10 weeks and transported after 1 week of recovery; intact male mice were transported at 10 weeks. All mice were habituated for 3 days before experimental procedures were carried out. It should be noted that the age of ovariectomy of the female mice was kept constant throughout the experiment. Additionally, the time of experiment post ovariectomy and transportation was also kept constant to minimise any confounding effects.
**PRIMARY NEURAL CULTURE**

Primary rat cortical and hippocampal neurons were seeded onto 0.2 mg/mL Poly-D-lysine (PDL; Sigma: P0899) coated 6-well Nunc plates or 60 mm Nunc dishes for biochemistry and RNA isolation or 18 mm coverglass (VWR international: 630-2200) for immunocytochemistry. Neurons were cultured in feeding media, where all reagents were purchased from Life Technologies: neurobasal medium (21103049) supplemented with 2% B-27™ (17504044), 0.5 mM L-glutamine (25030024) and 1% penicillin: streptomycin (pen/strep; 15070063). Cortical neuron cultures were maintained in the presence of 200 µM D, L-aminophosphonovalerate (D, L-APV; Abcam: ab120004) from DIV (days in vitro) 4 to ensure the reduction of cell death as a result of excessive calcium cytotoxicity by over active NMDA receptors in high density cultures (Srivastava et al., 2011); this is particularly advantageous for long-term culturing however, this was not necessary for hippocampal neuron cultures. Half media changes were performed twice a week until desired age of experimentation, DIV 25-29 (cortical) or DIV 20-21 (hippocampal); these ages were favoured as it has been suggested that from DIV 21, neurons express mature dendritic spines (Srivastava et al., 2008, 2010).

**ACUTE SLICE PREPARATION**

Mice, both male and OVX female, were anaesthetised with isoflurane, followed by decapitation. Brain tissue was then rapidly removed and sliced 350 µm thick coronally in carbo-oxygenated (95% oxygen, 5% carbon dioxide), ice cold cutting solution using a Leica VT1000 vibratome. Hippocampi were rapidly dissected from the slices and maintained in a recovery chamber with carbo-oxygenated Ringer’s solution for 1 hour at 32°C. Slices were then transferred into 6-well plates containing 5-10 mL of freshly carbo-oxygenated Ringer’s solution in the presence of various drugs. Slices were collected and frozen at -80°C until lysing. All reagent compositions can be found in Table 2.1.
Primary rat hippocampal neurons were transfected at desired ages by coupling target plasmid DNA with Lipofectamine 2000 (LF2K) (Life Technologies: 11668027). Pre-optimised concentrations of DNA were mixed with LF2K in Dulbecco’s Modified Eagle’s medium (DMEM; Sigma: D6421) pH buffered with 15 mM HEPES (Fisher Scientific Ltd: 10041703) and left in a 5% CO₂ incubator at 37°C for 20 minutes with open caps for the DNA-LF2K complex to form. The complex was thereafter, added dropwise to the neurons and left to incubate overnight (cortical neurons) or 3-4 hours (hippocampal neurons) at 37°C with 5% CO₂. Transfections were carried out in transfection media (neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine) in a new plate. Following transfection, neurons were returned to their old feeding media.

Enhanced Green Fluorescent Protein (eGFP) DNA constructs were used to visualise and isolate the structure of neurons including the dendritic spines for these investigations. Hippocampal neurons were transfected with peGFP-C1 (Clontech). 2µg eGFP-C1 was coupled with 4 µL LF2K and introduced to neurons between DIV 12-14, the neurons were then left for 5-7 days before pharmacological treatments were initiated between DIV 20-21.

All pharmacological treatments were carried out in artificial cerebral spinal fluid (aCSF; Table 2.1) for primary cortical and hippocampal neurons, and Ringer’s solution for hippocampal slices. Neurons were pre-treated in aCSF and slices were recovered in for 1 hour prior to application of the drug compounds (Figure 2.1). If inhibitors were relevant to the experiment, then neurons were pre-incubated for 30 minutes after which the inhibitors would be added for 30 minutes followed by the addition of vehicle or estradiol. For slices, the inhibitors were added after 1 hour recovery for 30 minutes. All compounds were diluted in dimethyl sulfoxide (DMSO) and, serially diluted to a 10x working concentration in aCSF (neurons) or Ringer’s solution (slices). Table 2.2 depicts the final concentrations used for each compound. The concentration used for estradiol
has previously been optimised to study rapid estrogenic signalling by our lab and many other groups. Vehicle controls were made up of solvent (DMSO) lacking the compounds and diluted as test compounds; the solvent was diluted to at least 0.1%. Treatments were allowed to continue for the specified times and immediately followed by lysing for biochemistry, or fixation for immunocytochemistry (ICC).

**Figure 2.1 Schematic of pharmacological treatment timeline in primary rat neurons and acute slice preparations from mice within this study.** Primary neurons and acute slice preparations were treated or recovered in artificial cerebrospinal fluid (aCSF) or Ringer’s solution, respectively. Pharmacological treatments commenced following this and after the appropriate time, primary neurons were either lysed for western blotting, or fixed for immunocytochemistry (ICC); acute slices were solely lysed for western blotting.

**LYSATE PREPARATION**

Whole cell lysates

Primary cortical and hippocampal neurons were collected in ice cold Triton-lysis buffer: 20 mM Tris pH 7.2; 150 mM NaCl; 1% Triton X-100; and 5 mM EDTA pH 8 in the presence of protease (AEBSF, aprotinin, leupeptin, pepstatin A), and phosphatase inhibitors (sodium fluoride, sodium orthovanadate, phosphatase cocktail 3 (Sigma: P0044)) and incubated on ice for 30 minutes. Lysates were
hereafter sonicated with 8-10 pulses at 40% power and centrifuged at 15’000 rpm for 15 minutes at 4°C to pellet out residual membranes, and supernatant collected.

For acute hippocampal slices, the dry weight was initially acquired per sample and lysed 10 x v/w in ice cold lysis buffer: 50 mM NaPO₄; 40 mM NaCl, 5 mM EDTA; 5 mM EGTA; 1% Tritr in the presence of the same protease and phosphatase inhibitors. Lysates were then sonicated with 20 pulses at 30% power, before being eluted for 2 hours on a rotor at 4°C. Hereafter, samples were centrifuged at 12’800 x g for 20 minutes at 4°C to pellet out residual membranes, and supernatant collected.

The total concentration of all samples was measured at 562 nm using the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific; 10678484) and approximately 5-20 µg protein was loaded for western blotting. Primary cortical and hippocampal neuron lysates were mixed with 2 x laemmli sample buffer (Bio-Rad Laboratories; 161-0737) with 355 mM β-mercaptoethanol and denatured for 5 minutes at 95°C. Alternatively, hippocampal slice lysates were mixed with NuPage LDS 4 x sample buffer (Thermo Scientific: NP0007) with 0.1 mM dithiothreitol (DTT) and denatured for 10 minutes at 70°C.

Crude synaptosomal preparation

Primary hippocampal neurons were collected in 150 µL ice cold homogenisation buffer: 320 mM sucrose; 5 mM Na₄P₂O₇; 1 mM EDTA pH 8; and 10 mM HEPES pH 7.4 in the presence of protease and phosphatase inhibitors and homogenised by passage through a 25-gauge needle 12 times. The lysate was centrifuged at 800 x g for 10 minutes at 4°C to yield the P1 fraction (nuclear and large organelles) and S1 fraction (extra-nuclear). A fraction of S1 was retained separately (30 µL) and the remainder was centrifuged at 15’000 x g for 20 minutes at 4°C to yield the P2 fraction (crude synaptosomes) and S2 fraction (cytosolic). The P2 fraction was resuspended in 30 µL ice cold RIPA buffer: 10 mM Tris pH 7.2; 150 mM NaCl; 1% Triton-x100; 0.1% SDS; 1% deoxycholate; and 5 mM EDTA pH 8 in the presence of protease and phosphatase inhibitors.
Western blotting

Primary cortical neuron lysates were separated on 4-20% acrylamide gels (Bio-Rad: 456-1094). Primary hippocampal neuron lysates and hippocampal slice lysates were separated on 10% in-house made acrylamide gels through electrophoresis. The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) at 78 mA for 990 minutes at 4°C. Subsequently, membranes were blocked for 1 hour in TBS-T (with 0.1% tween) containing 5% bovine serum albumin (BSA) (Sigma: A7906) followed by an overnight incubation of specific primary antibodies in the same blocking solution at 4°C with agitation. Following 3 x 15 minute TBS-T washes, specific secondary HRP antibodies were incubated in the blocking solution for 2 hours at room temperature. Finally, membranes were incubated in Clarity Western ECL substrate (Bio-Rad: 170-5061) for 5 minutes before protein detection using the ChemiDoc XRS+ imaging system (Bio-Rad: 170-8265) running ImageLab™ software version 5.2.1 (http://www.bio-rad.com/en-uk/product/image-lab-software) (Bio-Rad). All proteins of interest and puromycin, for SUnSET, were normalised to the house keeper β-actin whereas, the phosphorylation of proteins were normalised to its respective total protein. See Table 2.1 for the composition of all the solutions essential for western blotting, and Tables 2.3 and 2.4 for the antibodies used within this investigation.

QUANTIFICATION OF WESTERN BLOTS

All images were taken in the linear range, to allow an accurate representation of the data. Acquired images were exported for analysis as a Tagged Image File Format (TIFF) and subsequently analysed using Image Studio™ Lite software version 5.2 (https://www.licor.com/bio/products/software/image_studio_lite) (LI-COR). For an image, the integrated density (ID) was measured per band or smear (for Surface Sensing of Translation; SUnSET) and a background correction calculated per band. A narrow strip on the top and bottom of the box drawn around the band was used to calculate the background correction as the median of the pixels within the band. For SUnSET, the narrow strip selection was left and right of the selection box. These ID values were exported to
Microsoft Excel and the target protein was normalised to a housekeeper by dividing the ID value of the target protein by the ID value of the housekeeper, per condition within a biological replicate. The data set from all biological replicates within an experiment was then further normalised in the following way. Firstly, all ID values were summed for each condition across all biological replicates. Secondly, the ID values from each condition were then divided by these summed ID values. This produced a transformed set of data where each datum was relative to the sum of all conditions across the biological replicates. This method of normalisation was selected from Degasperi et al., (2014) to account for differences in antibody staining, washing and developing produced in the western blotting technique when comparing biological replicates from the same experiment across different western blots. Finally, the mean of all the vehicle ID values was taken and used to transform all the data points around the value of 1 to easily represent the fold change in protein expression after a drug treatment, for example: \((1/\text{mean of vehicles}) \times \text{each datum}\).

**RNA ISOLATION**

**RNA extraction**

Primary hippocampal neurons were collected in 1 mL TRIzol reagent (Life Technologies: 15596026) and stored at -80°C until processing. RNA was extracted according to the manufacturer’s protocol. Briefly, cells lysed in Trizol were thawed at room temperature (RT) and subsequently mixed with 100 µL 1-bromo-3-phenolpropane (BCP), followed by 15 minute incubation. This solution was centrifuged at 13’000 x g for 15 minutes at 4°C. The top aqueous phase was collected and transferred into new tubes, to which 500 µL isopropanol was added per sample to precipitate the DNA; these were incubated at RT for 10 minutes and then centrifuged as before. The isopropanol supernatant was discarded, and the RNA pellets washed with molecular grade 75% ethanol, then centrifuged for 13’000 x g for 5 minutes; this was repeated 3 times. The supernatants were discarded, and the pellets were left to air dry for approximately 10-15 minutes. Nuclease free H₂O was added to each sample;
absorbances were measured per sample using a Nanodrop spectrophotometer ND-1000.

**cDNA synthesis**

The RNA was then treated with TURBO DNA-free™ kit (Thermo Fisher: AM1907) to remove any residual genomic DNA according to the company’s manual. Firstly, total RNA from each extraction was incubated with 2 units of Turbo DNase in 1 x Turbo DNase at 37°C for 30 minutes. Next, DNAse Inactivation Reagent was added to the RNA and incubated at RT for 5 minutes. The mixture was then centrifuged at the max speed for 3 minutes and supernatant collected. Reverse transcription was carried out on the RNA using Superscript III™ Reverse Transcriptase (SSIII) (Thermo Fisher Scientific: 18080-044) according to the manufacturer’s protocol. The reaction was prepared in two steps: primarily, a mixture (A) was made containing RNA, random decamers and dNTPs, which was heated to 65°C for 5 minutes and cooled on ice for approximately 1 minute. Following this, a second mixture (B) was made containing 5 X First-Strand Buffer, DTT, RNaseOUT™ Recombinant Ribonuclease Inhibitor and SSIII. This was added to mixture A producing a 20 µg final volume reaction of: approximately 907 ng RNA; 5 µM random decamers; 500 µM dNTPs; 5 mM DTT; 40 units RNaseOUT; 200 units SSIII in 1 x First-Strand Buffer. The reaction was incubated in a GS4 Thermocycler (G Storm; Pendragon Scientific Ltd.: GT-40364) following a pre-set protocol as followed: 25°C for 25 minutes; 50°C for 60 minutes; 55°C for 30 minutes; and 70°C for 15 minutes. Finally, cDNA was diluted to approximately 6 ng/µL and stored at -20°C.

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

Expression of DLG4, GRIN2B and GRIA1 was assessed using RT-qPCR, to identify potential changes in transcript abundance following acute estradiol treatment. Primers were designed using Primer3 and purchased from Integrated DNA Technologies (Table 2.5). PCR amplification of cDNA was carried out in a total volume of 12 µL using HOT FIREPol DNA Polymerase (Solis BioDyne: 01-
This reaction contained ~27ng cDNA; HOT FIREPol DNA Polymerase 1x; and 200 nM primers, and it was carried in a QuantStudio 7 Flex and the QuantStudio Real-Time PCR Software (Thermo Fisher Scientific). The following protocol was used: 95°C for 15 minutes (hot start); 40 cycles of 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 20 seconds; and a continuous melting curve analysis of the amplicons from 60-95°C, which revealed single peaks, thus confirming specificity of the oligonucleotides used. Gene expression quantification was calculated relative to a standard curve of five 1:2 dilution points (quantity), and this value was further normalised to the geometric mean of the quantity of the three housekeeping/reference genes, per condition (ACTB, GUSB, B2M).

**IMMUNOCYTOCHEMISTRY**

Primary cortical or hippocampal neurons were washed in cold phosphate buffered saline (PBS) post treatment, but before fixation. Neurons underwent one of the following fixation methods: 10 minute fixation with 4% formaldehyde in a 4% sucrose in PBS solution at RT, then 10 minute fixation with 100% methanol on ice (double fixation) at RT; or 10 minute fixation with 4% paraformaldehyde in a 4% sucrose in PBS solution (single fixation; PFA-sucrose) at RT. Neurons were then washed 2 x in PBS and then permeabilised and blocked simultaneously in PBS containing 0.1% Triton-x100 and 2% Normal Goat Serum (NGS; Cell signalling: 5425S) for 1 hour at RT unless otherwise indicated. Subsequently, primary antibodies were added in PBS containing 2% NGS and left overnight at 4°C. This was followed by 3 x 15 minute PBS washes. The neurons were then incubated in Alexa Fluor® secondary antibodies in PBS containing 2% NGS for 1 hour at RT; dyes were selected according to species in the following wavelengths: 405; 488; 568; or 647, see Table 2.4. This was again followed by 3 x 15 minute PBS washes with a 5 minute incubation with PBS containing 0.1% the nuclear stain 4’,6-diamidino-2-phenylindole (DAPI) (Life Technologies: D1306) unless otherwise stated. Finally the neurons were mounted with ProLong™ Gold antifade mountant (Life Technologies: P36930). A list of primary antibodies used in this investigation can be found in Table 2.3.
DETECTION OF PROTEIN SYNTHESIS

Surface Sensing of Translation (SUnSET)

Puromycin (Sigma; P8833) is an aminonucleoside antibiotic produced by *Streptomyces alboniger*. It is a recognised protein synthesis inhibitor; the structure of puromycin is analogous to that of tRNA and typically incorporates onto nascent polypeptide chains thereby, inhibiting the elongation of amino acids and thus, the translation of proteins. Using this mechanism as an advantage, an assay termed surface sensing of translation, or SUnSET, was developed (Schmidt et al., 2009). This assay employs puromycin as an indicator of the rate at which protein synthesis is occurring within a cell, if it is used at a low concentration for a short period of time (Schmidt et al., 2009). This approach can be utilised to determine the effect of a pharmacological treatment on the rate of protein synthesis by adding puromycin just before the end of the treatment to allow incorporation into nascent polypeptide chains. Subsequently, an antibody against puromycin is used to detect the puromycin incorporation post lysing or fixation (Figure 2.2). The level of puromycin is indicative of the rate of protein synthesis occurring.

Puromycin treatments were added at the last 10 minutes (neurons) or the last 30 minutes (slices) of pharmacological treatments at the final concentrations of 10 and 5 µg/ml respectively (Figure 2.3) The concentrations and treatment times were previously optimised for primary neurons (Schmidt et al., 2009) and acute slice preparations (Ma et al., 2013) to directly visualise the rate of translation occurring using SUnSET. The neurons or slices were then lysed for biochemistry or PFA-sucrose fixed for ICC. An antibody against puromycin was used for western blotting (Kerafast: EQ0001; SUnSET-WB) or immunocytochemistry (Milipore: MABE343; SUnSET-ICC) to visualise puromycin labelled proteins.
Figure 2.2 Schematic of the Surface Sensing of Translation (SUnSET) assay to measure the rate of protein translation. Puromycin incorporates onto nascent polypeptide chains inhibiting the elongation of amino acids and therefore, the translation of proteins. When used at a low concentration for a short period of time, the rate of translation can be visualised by using an antibody against puromycin through western blotting or immunocytochemistry (ICC). Figure adapted from Goodman and Hornberger, (2013).

Figure 2.3 Schematic of puromycin (SUnSET assay) treatment timeline in primary rat neurons and acute slice preparations from mice within this study. Acute slices were recovered for 1 hour after which, if relevant to the experiment, specific inhibitors were added 30 minutes prior to the estradiol treatment (10 nM), which occurred over 2 hours. Puromycin was added in the last 30 minutes of the experiment at 5 µg/mL followed by lysing for western blotting. Primary
neurons were pre-treated in artificial cerebrospinal fluid (aCSF) for 1 hour followed by a 2 hour estradiol (10 nM) treatment. If inhibitors were relevant for the experiment, primary neurons were pre-treated for only 30 minutes and inhibitors added for further 30 minutes followed by a 2 hour estradiol treatment. Puromycin was added in the last 10 minutes of the experiment at 10 µg/mL followed by lysing for western blotting or fixing for immunocytochemistry (ICC).

**Fluorescent noncanonical amino acid tagging (FUNCAT)**

Fluorescent noncanonical amino acid tagging, or FUNCAT, is an alternative method to detect newly synthesised proteins using fluorescent labelling via copper catalysed click chemistry. The assay involves the presentation of azide-bearing or alkyne-bearing analogs of amino acids respectively, azidohomoalanine (AHA) or homopropargylglycine (HPG), into neuronal medium which, are then incorporated into nascent proteins. Referred to as methionine surrogates (tom Dieck et al., 2012), AHA or HPG is incorporated at methionine codons into live cells or slices and are subsequently covalently reacted to an alkyne or azide fluorophore, respectively, through a copper catalysed click reaction. Whilst SUnSET provides information about the rate (SUnSET-WB) or site (SUnSET-ICC) of translation, FUNCAT shows newly synthesised proteins. Thus, combining both techniques provides information about the amount of translation and where nascent proteins are targeted following a pharmacological treatment.

Primary cortical and hippocampal neurons were treated with 4 mM AHA (Life Technologies: C10102) for 2 hours following an optimisation experiment to illustrate the treatment time that would be optimal to visualise newly synthesised proteins along the dendrites. The concentration of AHA was chosen in accordance to the published protocol by Daniela Dieterich and Erin Schuman (tom Dieck et al., 2012). Additionally, the treatment time was chosen based on an optimisation on an earlier published paper (Dieterich et al., 2010). The concentration and treatment times were verified to determine if AHA staining could be visualised within secondary and tertiary dendrites (Appendix 3A). Primary neurons were initially pre-incubated in aCSF for 60 minutes then
treated simultaneously with estradiol and AHA; these were incubated for 2 hours at 37°C with 5% CO₂. The published protocol (tom Dieck et al., 2012) was followed with minor adjustments. Primary neurons were thereafter briefly washed in PBS-MC (PBS pH 7.4, 1 mM MgCl₂; 0.1 mM CaCl₂) followed by a 10 minute PFA-sucrose fixation at RT. These were washed 3 x in PBS pH 7.4 then simultaneously permeabilised and blocked in PBS containing 0.1% Triton-x100 and 2% NGS for 1.5 hours at RT. The neurons were washed 3 x in PBS pH 7.8 and incubated upside down in the FUNCAT reaction mix (PBS pH 7.8, 0.2 mM TBTA¹, 0.5 mM TCEP², 0.2 µM Alexa Fluor 555-Alkyne tag, 0.2 mM CuSO₄) overnight at RT. The following day, the neurons were washed face up 3 x for 10 minutes in FUNCAT wash buffer (PBS pH 7.8, 0.5 mM EDTA, 1% Tween-20) and 2 x for 10 minutes in PBS pH 7.4. The neurons were then incubated in primary antibodies overnight at 4°C and fluorescent secondaries as per the protocol described previously. For the purposes of these experiments, no DAPI incubation was implemented. The full protocol and recipe for all components can be found in tom Dieck et al. (2012). Figure 2.4 depicts the pharmacological treatment time-line.

¹ Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
² Tris-(2-carboxyethyl)phosphine hydrochloride
Figure 2.4 Schematic of azidohomoalanine (AHA) treatment and subsequent fluorescent non-canonical amino acid tagging labelling (FUNCAT) protocol timeline in primary rat neurons within this study. Primary neurons were pretreated in artificial cerebrospinal fluid (aCSF) for 1 hour. Following this, estradiol (10 nM) and AHA (4 mM) were simultaneously added onto the neurons for 2 hours. These were then fixed in 4% paraformaldehyde in a 4% sucrose in PBS solution (single fixation; PFA-sucrose) for 10 minutes and simultaneously permeabilised and blocked in PBS containing 0.1% Triton-x100 and 2% normal goat serum (NGS) for 1.5 hours. The FUNCAT reaction (PBS pH 7.8, 0.2 mM TBTA, 0.5 mM TCEP, 0.2 µM Alexa Fluor 555-Alkyne tag, 0.2 mM CuSO4) was then applied overnight to the neurons. Immunocytochemistry (ICC) was carried out the next day as normal.

**MICROSCOPY AND IMAGE ACQUISITIONS**

Epifluorescence imaging and confocal microscopy

Epifluorescence imaging was used for puromycin optimisation (Appendix 1C). Representative images were taken on a Zeiss Axio Imager Z1 acquired using a 40x objective (Numerical Aperture [N.A] 0.8). Representative images for AHA
treatment optimisation (Appendix 3A) were taken using a Leica SP5 inverted confocal microscope acquired with 20x objective (N.A. 0.7). Confocal images were taken as a Z-series (z-step= 0.5 µm). All representative images from Chapter 4 and Chapter 5 (Figures 5.4-5.5) and Appendix 3B were acquired by a Nikon A1R inverted confocal with 60x oil-immersion objective (N.A. 1.4) at the Wolh Cellular Imaging Centre. All images were taken as a Z-series (z-step= 0.15 µm). Both epifluorescent and confocal images were exported to Fiji (https://fiji.sc/) where two-dimensional maximum projections reconstructions were generated. The appendix images (Appendix 1C, 3A-B) were appropriately adjusted for brightness and contrast or pseudocoloured to emphasise areas of high staining intensities on Fiji. All confocal images for Chapters 4 and 5 were further processed using MetaMorph software (Universal Imaging Corporation). Each image was background subtracted via a statistical correction. All images were taken in the linear range, to allow an accurate representation of the data. The image acquisition parameters were the same for all images within an experiment to allow comparison.

Structural Illumination Microscopy (SIM)

SIM imaging for Chapter 5 (Figures 5.8-10) was performed on a Nikon iSIM super-resolution microscope at the Wolh Cellular Imaging Centre. Images were acquired using a 100x oil-immersion objective (N.A. 1.49TIRF) with 51 z-steps (z-step= 0.12 µm). Raw images were acquired and deconvolved using a 3D blind algorithm specific to the iSIM to increase resolution using the NIS-Elements Advanced Research software (Nikon, version 5.01.00). All images were exported to Fiji where maximum projections were generated and subsequently analysed for puncta size, area and co-localisation, detailed below. The image acquisition parameters were the same for all images within an experiment.
Quantitative analysis of dendritic spine morphology and immunofluorescence

Dendritic spine morphology was all carried out using MetaMorph as previously detailed (Srivastava et al., 2011) and all subsequent analysis of the data was done on Microsoft Excel. The analysis was carried out using images taken on the A1R inverted confocal with a 60x oil-immersion objective (N.A. 1.4), with at least 3 to 4 neuronal cells per condition over 4 biological replicates. Figure 2.5 depicts the workflow of the analysis. Per neuronal cell, two separate pieces of dendrites, secondary and tertiary branches, were traced manually to total to approximately 100 µm in length (Figure 2.5A, B, G). Using these lengths, regions were manually traced on either side of the dendrite separating the dendritic spine regions on either side along the dendrites (Figure 2.5C). The dendritic spines on either side were then thresholded by MetaMorph in a manner that outlines the spines and preserves the morphology (Figure 2.5D). Each spine was then saved as a separate region to assess morphologies per condition (Figure 2.5E+F). This method was also used to assess the average intensity of PSD-95, GluN2B and AHA within spines following estradiol treatment. The spine regions created as detailed above were then projected into the protein of interest/AHA channels of the corresponding image set to assess the average intensity within spines. Additionally, from the same 100 µm regions, the dendrites were thresholded by MetaMorph (Figure 2.5H) and the dendritic region created (Figure 2.5I). The dendritic regions were then placed in the channel of interest to measure average intensity. Figures 2.5J-L depict exemplar spine morphologies that can typically occur: stubby structure (J); lollipop structure (K); and mushroom structure (L); the spines come from a DIV 27 primary cortical neuron imaged by SIM by Iain Watson, a member of the Srivastava lab.
Figure 2.5 Analysis of dendritic spine morphology in cultured primary neurons using Metamorph. (A) Confocal image (60x) of DIV 21 cultured hippocampal neuron expressing eGFP. Dendritic spine analysis, (B) Dendrites were traced manually totalling to 100 µm and (C) used to create regions either side of the dendrite. (D) Dendritic spines on either side were then thresholded by Metamorph and (E) recognised as regions of interests to assess number and morphology. Dendrite analysis, (G) As B, the dendrite was traced manually and (H) thresholded by Metamorph. (I) Dendritic region was isolated and created. (J-L) Super resolution images kindly provided by Iain Watson (Srivastava lab) acquired by SIM (100x) of typical dendritic spine morphology found on primary neurons. From left to right: stubby structure; lollipop structure; and mushroom structure. Yellow dashed box denotes section of dendrite displayed in inset. Scale bars = 100 µm for (A); 10 µm for (B-I); 1 µm for (J-L).
Analysis of new protein expression using FUNCAT

As above, the analysis of AHA along dendrites and within dendritic spines was carried out using images taken on the A1R inverted confocal with a 60X objective. For each image, the dendrite and spine regions would be identified and isolated and placed into the AHA channel to measure average intensity of AHA. As AHA intensity represents newly synthesised proteins, there can be variability to the staining as AHA-tagged nascent proteins can also be those that have been translated in the cell soma. Inconsistent measurements incorporating the primary dendrite from the soma compared to tertiary dendrite could confound any changes between conditions. Thus, per cell, secondary and tertiary dendrites were equally chosen for analysis as depicted in Figure 2.6. This was kept consistent between conditions and over biological replicates.

**Figure 2.6** Analysis parameters for AHA-tagged newly synthesised proteins in cultured primary neurons. Confocal image (20x) of DIV 21 cultured hippocampal neuron expressing eGFP, treated with AHA, and subsequently tagged with Alexa Fluor 555-Alkyne tag. Only secondary and tertiary dendrites were consistently chosen and traced per cell for analysis of AHA intensity across conditions and over biological replicates. Yellow dashed box denotes section of dendrite displayed in inset. Red dashed boxes denote regions of measurement. Scale bars = 100 µm, inset 50 µm.
Analysis of puncta co-localisation

Images stained for neuronal marker MAP2, puromycin (SUnSET-ICC) and ribosomal proteins S10 (RPS10) acquired from SIM and processed through deconvolution were measured for co-localisation in Fiji. For each cell, 50 µm in length was traced along two or more separate pieces of dendrites within the MAP2 channel. As dendritic spines cannot be visualised with MAP2 staining, 2.5 µm either side of the dendrite was traced to create ‘spine regions’ that would encompass all the dendritic spines along that portion of that dendrite. Specifically 2.5 µm either side of the dendrite was selected, as dendritic spine lengths have typically been reported to average between 1-1.5 µm in length in 2-3 week old cultures (Boyer et al., 1998). Capturing 2.5 µm either side of the dendrite allows for any variability within dendritic spine lengths ensuring some spines are not cut off within the analysis. It is important to emphasise that these are crude analyses of ‘spine regions’, as the spine regions are estimations using the MAP2 staining as opposed to identified spines via diffused GFP overexpression. Thus, the ‘dendrite region’, the ‘spine region’, and these regions together termed ‘crude synaptic region’ were all assessed for puncta co-localisation (Figure 2.7). Images in the RPS10 staining channels were thresholded and particle analysis was performed on the regions of interest (ROI) within the puromycin channel to determine how many puromycin puncta would co-localise with RPS10 puncta. Expression within a region of interest was determined by whether staining intensity was greater than 25% of the maximum staining intensity.

The approach of co-localisation adapted within the current study assumes that the images acquired have little background noise therefore, thresholding puncta can be problematic if they are not clearly identifiable. An alternative method to analyse colocalization is Pearson’s co-localisation co-efficient, which can be used to determine the degree of linear relationship of fluorescent intensities between two different channels (Dunn et al., 2011). A disadvantage is that it is sensitive to background therefore, Pearson’s co-localisation co-efficient may identify a false correlation in images with high backgrounds (Adler & Parmryd, 2010). Nevertheless, if the images have very little background, the sensitivity in
identifying a correlation is good. Pearson’s co-localisation co-efficient assumes a simple linear relationship and is not sensitive to differences in signal intensity of different channels (Dunn et al., 2011). Indeed, there are other methods to analyse co-localisation such as Manders Overlap Co-efficient as it measures the fraction of pixels with positive values that occur in both channels thereby, measuring absolute intensities; these methods are reviewed in Adler & Parmryd (2010). It is key to note that each method has its advantages and disadvantages and that the quality of image is also a determinant of which method may be better. It may be advantageous to employ multiple methods to report co-localisation to avoid reporting false-positive correlations. Furthermore, determining co-localisation can be problematic when puncta are numerous and overlapping. This can lead to interpretations that puncta may be clustering with each other or with puncta from other proteins when in fact they may not be, leading to incorrect conclusions. Moreover, if puncta from one channel are overlapping, this can lead to interpreting this as an individual punctum rather than multiple puncta. Images are acquired from a 3D environment and processed to produce 2D images whereby, a 2D co-localisation analysis is performed where numerous information may be lost or misinterpreted. A 3D analysis tool should be considered such as, the novel plugin DiAna for ImageJ as an alternative method for distance analysis in 3D (Gilles et al., 2016). The plugin allows to report in depth how much two objects may co-localise measuring: the distance from the centre of each puncta; the distance from the edge of each puncta; the distance from the centre of each puncta; and how much both puncta overlap (Figure 2, Gilles et al., 2016) providing a greater wealth of information.

Figure 2.7 Analysis parameters for determining spine and dendritic regions for puncta analysis in MAP2 positive neurons. Dendritic regions were traced, and regions 2.5 µm either side were selected to encompass dendritic spines in MAP2 positive neurons. Collectively, these were termed ‘crude synaptic
regions’ encompassing both dendrites and dendritic spine regions. Puncta and puncta co-localisation were measured in crude synaptic regions, dendrites and dendritic spines regions. The black lines represent what regions were measured within each heading. This figure was kindly provided by Iain Watson.

STATISTICS

All statistical analysis was performed on Graphpad Prism 6. For all graphs, bars represent the mean average and error bars are presented as standard error of the mean (SEM). To identify differences between vehicle and pharmacological treatments, unpaired Student’s t-test with Welch’s correction was performed; the Mann-Whitney test was chosen for non-parametric data. A one-way ANOVA was employed for comparisons between multiple conditions; Bonferroni’s post-hoc analyses were performed to correct for multiple comparisons of parametric data. The Kruskal Wallis test by ranks corrected by Dunn’s multiple comparisons test was performed on non-parametric data. An online Grubb’s test was used to identify any significant outliers (https://www.graphpad.com/quickcalcs/grubbs1/).
Table 2.1 Composition of common solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary neuron treatments</td>
<td>artificial cerebral spinal fluid (aCSF) (in mM): 125 NaCl; 2.5 KCl; 26.2 NaHCO₃; 1 NaH₂PO₄; 11 glucose; 5 HEPES pH 7.4; 2.5 CaCl₂; 1.25 MgCl₂; 0.2 APV (no APV needed for hippocampal neurons)</td>
</tr>
<tr>
<td>Acute slice preparation</td>
<td>Cutting solution (in mM): 87 NaCl; 3 KCl; 7 MgCl₂; 1.25 NaH₂PO₄; 0.5 CaCl₂; 50 sucrose; 25 glucose; 25 NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>Ringer’s solution (in mM): 126 NaCl; 10 glucose; 2 MgCl₂; 2 CaCl₂; 2.5 KCl; 1.25 NaH₂PO₄; 1.5 mM C₃H₅NaO₃; 1 L-glutamine; 2.6 NaHCO₃</td>
</tr>
<tr>
<td>Western blotting</td>
<td>Running buffer (5X) pH 8.3 (in g): 15.1 tris(hydroxymethyl) aminomethane; 72 glycine; 5 SDS – diluted to 1X</td>
</tr>
<tr>
<td></td>
<td>Transfer buffer (10X) pH8.7 (in g): 30.3 trizma base; 151.6g glycine – diluted to 1X with 20% methanol</td>
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<tr>
<td></td>
<td>TBS (10X) pH 7.5 (in g): 60 tris(hydroxymethyl) aminomethane; 87 NaCl – diluted to 1X with 0.1% Tween20 (TBS-T)</td>
</tr>
<tr>
<td>ICC</td>
<td>PBS pH 7.6 (in mM): 137 NaCl; 2.7 KCl; 10 NaH₂PO₄; 1.8 KH₂PO₄</td>
</tr>
</tbody>
</table>

Table 2.2 Drug compounds, and their final concentrations, used in pharmacological treatments in both primary neurons and acute slices.

<table>
<thead>
<tr>
<th>Drug/compound</th>
<th>Company</th>
<th>Catalogue no.</th>
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<td>Estradiol</td>
<td>Sigma</td>
<td>E8875</td>
<td>10 nM</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Cell Signaling</td>
<td>9904</td>
<td>10 µM (neurons)</td>
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<td></td>
<td></td>
<td>1 µM (slices)</td>
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<td>Anisomycin</td>
<td>Sigma</td>
<td>A9789</td>
<td>40 µM</td>
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<td>Actinomycin D</td>
<td>Sigma</td>
<td>A4262</td>
<td>20 µM (neurons)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>25 µM (slices)</td>
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Table 2.3 List of primary antibodies used within the investigation. Abbreviations: WB = western blotting; ICC = immunocytochemistry.

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<th>Antibody</th>
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<th>ICC</th>
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<td>-</td>
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<td>Biolegend</td>
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<td>Rabbit</td>
<td>Synaptic Systems</td>
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<td>1:2000</td>
<td>-</td>
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<td>MABE343</td>
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<td>1:5000</td>
<td>-</td>
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<td></td>
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<td>Kerafast</td>
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<td>Catalog Number</td>
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Table 2.4 List of secondary antibodies used within the investigation.
Abbreviations: WB = western blotting; ICC = immunocytochemistry.

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Table 2.5 Primer sequences used within the investigation.

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CHAPTER 3

ACUTE ESTROGEN SIGNALLING REGULATES SYNAPTIC PROTEIN EXPRESSION IN A SEXUALLY DIMORPHIC MANNER

SUMMARY

Employing acute hippocampal slice preparations from both male and OVX female mice, this chapter aimed to biochemically investigate the expression profile of key excitatory and inhibitory synaptic proteins following a 2 hour estradiol treatment. The data demonstrated that estradiol regulates the expression of excitatory and inhibitory synaptic proteins differentially. Crucially, this chapter provides an insight into how estradiol regulates synaptic protein expression in a sexually dimorphic manner. This is the first study to investigate and report protein expression differences in both sexes using a wide array of key synaptic proteins. As sex differences are observed in hippocampal morphology, functioning, physiology and hippocampal-driven behaviour, dissecting the mechanisms by which estradiol contributes to these sex differences may provide valuable insights into understanding the biology of diseases and the need for sex specific treatments.

INTRODUCTION

Estrogens, particularly estradiol, have repeatedly been implicated in contributing to long-lasting influences over learning and memory (Frick, 2015; Srivastava et al., 2013; Woolley, 2007). These long-lasting effects infer that an alteration of
specific components of the neuronal architecture is occurring. The mechanisms underlying these long-lasting changes are, in part, orchestrated by estradiol’s ability to regulate synaptic plasticity in the hippocampus (Woolley, 2007). Evidence suggests that estradiol is able to trigger alterations in distinctive elements of the hippocampal circuitry such as, modulating excitatory synaptic transmission and dendritic spine density (Liu et al., 2008; Srivastava et al., 2013; Woolley, 2007). However, the molecular signature driving estradiol-mediated synaptic plasticity, and enhanced cognition, has yet to be fully elucidated.

A myriad of studies has demonstrated that estradiol regulates plasticity in the mammalian hippocampus employing a number of mechanisms. For example, estradiol has been shown to increase the formation of new dendritic spines in the hippocampus in vivo (Gould et al., 1990; Woolley and McEwen, 1994) and in cultured hippocampal neurons (Korkotian and Segal, 2001; Murphy and Segal, 1996; Murphy et al., 1998a). Recently, it has been found that estradiol increases spinogenesis rapidly, within minutes, in the hippocampus (Inagaki et al., 2012; Phan et al., 2012; Tuscher et al., 2016b) and the cortex (Inagaki et al., 2012; Sellers et al., 2015b; Srivastava et al., 2008). Estradiol also increases excitatory synapses specifically in the dorsal hippocampus (Woolley and McEwen, 1992), and potentiates EPSPs in excitatory synapses within the hippocampus of both sexes (Teyler et al., 1980; Wong and Moss, 1992). It has recently been demonstrated that this is mediated by distinct ERs in the male and female rat hippocampus (Oberlander and Woolley, 2016). Furthermore, estradiol enhances LTP and NMDA mediated synaptic transmission in the hippocampus (Good et al., 1999; Smith and McMahon, 2006; Warren et al., 1995; Woolley et al., 1997). This augmentation in hippocampal LTP has been observed as early as 2 hours following the activation of ERβ in the male mouse hippocampus (Kramár et al., 2009; Liu et al., 2008). Estradiol-mediated enhancement of LTP is deficient in ERβ KO mice (Liu et al., 2008), indicating a critical role for this receptor. Additionally, estradiol has been shown to modulate LTD, via ERα, in the male rat hippocampus (Mukai et al., 2007). Thus, it is appreciated that estrogens regulate complex actions at the synaptic architecture leading to enhanced synaptic transmission in the hippocampus and several studies have started to uncover the molecular mechanisms that may underlie this.
A potential mode of action estradiol may employ to regulate synaptic plasticity is by modulating the proteins prominently found at synapses. Excitatory and inhibitory synapses are organised on pre- and post-synaptic sides to induce specific protein and receptors that establishes their identity (Gerrow et al., 2006; McAllister, 2007). The majority of studies have focused on the estrogenic-regulation of excitatory post-synaptic proteins. Studies from our lab have shown that key proteins present at excitatory PSDs such as, PSD-95, adhesion protein Neuroligin 1 (Nlgn1) and GluN1 are rapidly recruited to nascent synapses following 30 minute estradiol treatment in primary rat cortical neurons (Sellers et al. 2015b). This suggests that estradiol-induced dendritic spines encompass the molecular machinery needed for synaptogenesis. Additionally, our collaborators have previously shown an increase in PSD-95 and GluA1 expression levels following a 4 hour in vivo application of estradiol in the OVX female rat, which was recapitulated in the female mouse hippocampus via ERβ (Liu et al., 2008). These studies thus, indicate that estradiol can rapidly reorganise excitatory synapses by increasing the expression of key post-synaptic proteins.

There is evidence that post-synaptic changes are often accompanied by pre-synaptic changes. Limited studies have looked at pre-synaptic changes in response to estradiol. Nevertheless, the enzyme that drives the synthesis of estradiol, aromatase, is localised pre-synaptically (Cornil et al., 2012; Naftolin et al., 1996; Remage-Healey et al., 2011; Srivastava et al., 2010) in addition to receptors ERα (Hart et al., 2007), ERβ (Milner et al., 2005) and GPER1 (Waters et al., 2015). Pre-synaptic proteins have been shown to fluctuate in expression during the estrous cycle (Crispino et al., 1999). Consistent with this, a number of studies have reported changes in pre-synaptic protein expression following chronic exposure to estradiol. Increases in vesicular glutamate transporter 1 (vGlut1) (Jelks et al., 2007), syntaxin (Brake et al., 2001) and synaptophysin (Rune et al., 2002) have previously been observed. Expression changes in a more rapid time-frame have also been reported in both synaptophysin (Liu et al., 2008) and synaptosomal associated protein 25 (SNAP 25) (Pechenino and Frick, 2009). Studies from Catherine Woolley’s lab have implicated a pre-synaptic mechanism contributing to estradiol-mediated EPSCs. Estradiol was found to increase the probability of glutamate release at synapses within 30 minutes through ERβ in the OVX female hippocampus (Smajkalova and Woolley, 2010).
Moreover, studies have shown that estradiol increases the frequency of multiple synapse boutons in hippocampal CA1 (Woolley et al., 1996; Yankova et al., 2001), where pre-synaptic boutons can synapse with multiple dendritic spines (Harris, 1995). Conversely, aromatase inhibition decreases the number of presynaptic boutons (Kretz et al., 2004). Interestingly, this also decreased synapses on dendritic spines but not those on the dendritic shaft suggesting a consequential post-synaptic effect. These studies propose that estradiol is able to mediate changes at excitatory pre-synaptic terminals that may contribute to post-synaptic changes and consequently, synaptic neurotransmission in the hippocampus.

 Estradiol’s influence on excitatory neurotransmission in the hippocampus is well documented whereas, much less is known about its role in regulating inhibitory neurotransmission. Nevertheless, ERα (Milner et al., 2001; Murphy et al., 1998b), ERβ (Milner et al., 2005) and GPER1 (Waters et al., 2015) have all been localised to interneurons within the rodent hippocampus suggesting estrogenic signalling can be mediated within inhibitory neurons. Previous studies have demonstrated a decrease in pre-synaptic glutamic acid decarboxylase 65 and 67 (GAD-65/67) protein expression and GAD positive interneurons following 12 and 24 hour estradiol treatments, respectively (Murphy et al., 1998b). Estradiol has also been shown to differentially regulate their respective mRNA levels in distinct brain regions (McCarthy et al., 1995). Given that GAD-65/67 is responsible for GABA synthesis it is feasible that estradiol may be regulating inhibitory neurotransmission. In support, previous research has shown that estradiol (10 or 100 nM) suppressed inhibitory neurotransmission in OVX female, but not male, hippocampus through ERα within 10 minutes (Huang and Woolley, 2012). A follow up study suggested a pre-synaptic mechanism underlying this estradiol-induced inhibition involving group I mGluR1 signalling inducing a subsequent suppression of GABA release (Tabatadze et al., 2015). To fully understand how estradiol may be interacting with proteins inhabiting inhibitory synapses, our collaborators investigated the relationship of estradiol with postsynaptic scaffolding protein gephyrin and its interactions with various GABA-A receptors (GABAAR). They have recently shown that 2 hour estradiol treatment reduced the stability of inhibitory synapses, with a concurrent reduction in the amplitude of miniature inhibitory synaptic currents (mIPSCs) and spontaneous inhibitory
synaptic currents (sIPSCs) in primary rat cortical neurons and male mouse hippocampal slice preparations, respectively (Mukherjee et al., 2017). Critically, they report that estradiol disrupted synaptic clusters of GABA\(\alpha\)R synaptic subunits alpha-2 (\(\alpha_2\)) and gamma-2 (\(\gamma_2\)) with gephyrin within the same timeframe. Additionally, they showed that estradiol had no effect on the synaptically localised alpha-1 (\(\alpha_1\)) subunit. These data suggest that estradiol may interfere with the efficacy of inhibitory transmission by disrupting synaptic stability of GABA\(\alpha\)R-gephyrin synapses. Nevertheless, more studies are required to confirm this and to fully understand estradiol's role in inhibitory neurotransmission. Taken together, these studies highlight the complexity of estradiol's effect on the synaptic plasticity and that estradiol could modulate synaptic inhibition prior to, in concert with, or as a consequence with the changes observed at postsynaptic sites of excitatory neurons.

This chapter reports the expression profile of a number of key excitatory and inhibitory synaptic proteins following a 2 hour DMSO or estradiol treatment in acute hippocampal slices from both male and OVX female mice. Investigating how estradiol influences prominent synaptic proteins found at both excitatory and inhibitory synapses can provide an insight into a novel way estradiol may be mediating neuronal transmission in the hippocampus, which may contribute to enhanced hippocampal synaptic plasticity.

**RESULTS**

**Estradiol increases the expression of key excitatory post-synaptic proteins in both male and OVX female hippocampus**

Increased expression levels of post-synaptic proteins predominantly present at excitatory PSDs has previously been reported within 4 hour estradiol treatment *in vivo* (Liu et al., 2008). Given the importance of PSD-95 and GluA1 in hippocampal synaptic functioning and plasticity (Sheng and Kim, 2011), the current study sought to determine whether estradiol increased expression of these proteins, in addition to various NMDAR subunits, within an earlier timeframe. Acute hippocampal slices from 10-12 week old male and OVX female
mice were treated with DMSO or estradiol for 2 hours. Hippocampi from each animal were divided by hemisphere into the different treatment conditions, DMSO and estradiol, per slice. This set up allowed an assessment of the effect of estradiol compared to a control within each animal. The slices were processed for western blotting (as described in Chapter 2) and the expression profile of PSD-95, the AMPAR subunit GluA1 and the NMDA receptor subunits GluN1, GluN2A and GluN2B were assessed in response to estradiol treatment. A significant increase in PSD-95 expression levels was observed in slices treated with estradiol compared to DMSO treated slices in the male ([mean ± SEM] vehicle 1.000 ± 0.02, E2 1.371 ± 0.02; t=10.71, df=8.00, p<0.0001, n=5 per condition; Figure 3.1A) and OVX female ([mean ± SEM] vehicle 1.000 ± 0.03, E2 1.213 ± 0.03; t=5.961, df=8.00, p=0.0003, n=5 per condition; Figure 3.1C) hippocampus. Estradiol also increased the expression levels of GluA1 in males ([mean ± SEM] vehicle 1.000 ± 0.03, E2 1.198 ± 0.03; t=4.714, df=10.00, p=0.0008, n=6 per condition; Figure 3.1B) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.02, E2 1.311 ± 0.03; t=9.281, df=8.00, p<0.0001, n=5 per condition; Figure 3.1D) within the same time-frame. These results mirror the findings of Liu et al. (2008) and suggest that estradiol is increasing the expression of these proteins in a more rapid time-frame than previously reported.

No significant change in GluN1 expression levels was observed between male hippocampal slices treated with DMSO or estradiol ([mean ± SEM] vehicle 1.000 ± 0.04, E2 1.030 ± 0.04; t=0.4757, df=8.00, p=0.6470, n=5 per condition; Figure 3.2A). Conversely, estradiol increased GluN1 expression levels in OVX female slices ([mean ± SEM] vehicle 1.000 ± 0.13, E2 2.490 ± 0.45; t=3.164, df=5.842, p=0.0202, n=6 per condition; Figure 3.2D) within the same time-frame. The observed increase in GluN1 expression in OVX female hippocampus is contradictory to the findings of Liu et al. (2008) where estradiol induced no change in GluN1 expression levels in OVX female rat. Expression levels GluN2A were found to be increased in both male ([mean ± SEM] vehicle 1.000 ± 0.07, E2 1.444 ± 0.07; t=4.207, df=10.00, p=0.0018, n=6 per condition; Figure 3.2B) and OVX female ([mean ± SEM] vehicle 1.000 ± 0.06, E2 1.434 ± 0.06; t=5.312, df=8.00, p=0.0007, n=5 per condition; Figure 3.2E) estradiol treated hippocampal slices compared to vehicle. Comparably, estradiol also induced an increase in GluN2B expression levels in both male ([median] vehicle 1.028, E2 1.290; u=0,
p=0.0079, n=6 per condition; **Figure 3.2C** and OVX female ([mean ± SEM] vehicle 1.000 ± 0.07, E2 1.280 ± 0.07; t=2.882, df=8.00, p=0.0204, n=5 per condition; **Figure 3.2F**) hippocampal slices. Again, the increase in GluN2B expression contradicted with that of Liu et al.'s (2008).

Taken together, these data suggest that estradiol rapidly increases the expression of key proteins highly abundant at the PSDs of excitatory synapses in both the male and OVX female mice hippocampus. These results highlight a key event estradiol acutely promotes that may contribute to mediate hippocampal synaptic plasticity. More so, that estradiol may use different mechanisms within the male and OVX female hippocampus to mediate this.
Figure 3.1 Estradiol acutely increases PSD-95 and GluA1 expression in the male and ovx female hippocampus. A-D, Representative western blots and quantification of male (A+B) and ovx female (C+D) acute hippocampal slices prepared from 10-12 week old mice and treated with vehicle or estradiol (10 nM, 2 hours). Slices were processed for western blotting and probed for PSD-95 and GluA1 and normalised to housekeeper, β-actin. Estradiol increased expression levels of PSD-95 in the male (A) and ovx female (C) hippocampus within 2 hours; n=5-6 per condition. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; *** p = <0.001, **** p = <0.0001.
Figure 3.2 NMDAR subunits are increased in the male and ovx female hippocampus following 2 hour estradiol treatment. A-F, Representative western blots and quantification of male (A-C) and ovx female (D-F) acute hippocampal slices prepared from 10-12 week old mice and treated with vehicle or estradiol (10 nM, 2 hours). Slices were processed for western blotting and probed for GluN1, GluN2A and GluN2B and normalised to housekeeper, β-actin. Estradiol had no effect on GluN1 expression levels in the male hippocampus (A) but was increased in the ovx female (D) hippocampus after 2 hours. Expression levels of both GluN2A and GluN2B were increased within the same time-frame in both male (B+C) and ovx female (D+E) hippocampus; n=5-6 per condition. Unpaired student’s t-test with Welch’s correction was used with parametric data sets; Mann-Whitney test was used with non-parametric data sets; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, *** p = <0.001, ns = not significant.
Estradiol induces a sex specific difference in the expression of post-synaptic inhibitory proteins

Effective communication between neurons involves the synchronisation of excitatory and inhibitory neurotransmission. Previous research has suggested that acute estradiol treatment disrupts the stability of inhibitory synapses (Mukherjee et al., 2017) and suppresses inhibitory neurotransmission in a sex-specific manner (Huang and Woolley, 2012; Tabatadze et al., 2015). This suggests that estradiol may influence synaptic inhibition in concert with, or as a consequence of, changes it exhibits at excitatory synapses. To understand whether estradiol-mediated increases seen in synaptic proteins is restricted to excitatory synapses, the expression profile of key proteins found at inhibitory synapses were assessed within the same time-frame.

As above, male and OVX female hippocampal slices were treated with DMSO or estradiol for 2 hours and processed for western blotting. The expression levels of scaffolding protein gephyrin and adhesion protein neuroligin-2 (Nlgn2) were assessed in response to estradiol. Estradiol significantly increased expression levels of gephyrin in the male ([mean ± SEM] vehicle 1.000 ± 0.01, E2 1.218 ± 0.01; t=14.63, df=8.00, p<0.0001, n=5 per condition; Figure 3.3A) but not the OVX female ([mean ± SEM] vehicle 1.000 ± 0.06, E2 0.9673 ± 0.06; t=0.3806, df=8.00, p=0.7134, n=5 per condition; Figure 3.3D) hippocampal slices. Critically, no significant difference in Nlgn2 expression levels was found in either male ([mean ± SEM] vehicle 1.000 ± 0.03, E2 1.088 ± 0.03; t=1.800, df=8.00, p=0.1096, n=5 per condition; Figure 3.3B) or OVX female ([mean ± SEM] vehicle 1.000 ± 0.07, E2 0.8675 ± 0.07; t=1.362, df=8.00, p=0.2104, n=5 per condition; Figure 3.3E) hippocampus. Additionally, given that gephyrin has a critical role in GABA\textsubscript{A}R clustering and stabilisation (Tyagarajan and Fritschy, 2014), the expression of alpha-1 (α1) subunit of GABA\textsubscript{A}R was assessed in male hippocampal slices alone for preliminary testing. GABA\textsubscript{A}R α1 was found to be significantly increased in estradiol treated hippocampal slices compared to vehicle ([mean ± SEM] vehicle 1.000 ± 0.04, E2 1.248 ± 0.04; t=4.083, df=4.00, p=0.0151, n=3 per condition; Figure 3.3C).
**Figure 3.3** Estradiol differentially regulates key inhibitory post-synaptic proteins in the male and ovx female hippocampus. A-E, Representative western blots and quantification of male (A-C) and ovx female (D+E) acute hippocampal slices prepared from 10-12 week old mice and treated with vehicle or estradiol (10 nM, 2 hours). Slices were processed for western blotting and probed for Gephyrin and Neurologin-2 (Nlgn2) and normalised to housekeeper, β-actin. For preliminary investigation, GABAAR alpha1 subunit (GABAAR α1) was assessed only in male slices. Estradiol increased Gephyrin expression in the male (A) but not ovx female (D) hippocampus within 2 hours. No expression change was observed in Nlgn2 levels in neither males (B) nor ovx females (E) following estradiol treatment within the same time-frame. Estradiol increased GABAAR α1 expression in the male hippocampus; n=5-6 per condition, n=3 for GABAAR α1. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p =0.05, **** p = <0.0001, ns = not significant.
This is the first study to report the effects of estradiol on both Nlgn2 and gephyrin in the mouse hippocampus. These data show that estradiol increases gephyrin and GABA\(\alpha_1\)R expression levels in the male hippocampus. A distinct effect is observed in the OVX hippocampus suggesting estradiol may behave differently at inhibitory synapses in the females. Further studies are required to understand the effect of estradiol on the expression level of GABA\(\alpha_1\)R in the OVX female hippocampus.

**Excitatory pre-synaptic proteins are differentially altered in response to estradiol**

Previous research has demonstrated that estradiol engages a presynaptic mechanism to potentiate excitatory neurotransmission (Smejkalova and Woolley, 2010). Interestingly, estradiol has previously been shown to enhance synaptophysin in a region specific manner (Liu et al., 2008), but the effect of acute estradiol on other pre-synaptic proteins have not been investigated. To this end, the expression profile of neurotransmitter release regulator synapsin 1, SNAP 25 and synaptic vesicle protein 2A (SV2A) were all assessed in acute hippocampal slices from adult male and OVX female mice following a 2 hour estradiol treatment. Synapsin 1 expression was not significantly different between DMSO treated and estradiol treated hippocampal slices from either males ([mean ± SEM] vehicle 1.000 ± 0.02, \(E2\) 0.9915 ± 0.02; \(t=0.2509, df=8.00, p=0.8082, n=5\) per condition; **Figure 3.4A**) or OVX females ([median] vehicle 1.004, \(E2\) 0.8956; \(u=3, p=0.2000, n=4\) per condition; **Figure 3.4D**). Sex specific difference in expression was observed in both SNAP 25 and SV2A. Estradiol had no effect on SV2A expression in the male hippocampus ([mean ± SEM] vehicle 1.000 ± 0.03, \(E2\) 0.9766 ± 0.03; \(t=0.6264, df=8.00, p=0.5485, n=5\) per condition; **Figure 3.4B**) whereas, it decreased SV2A expression in the OVX female hippocampus ([mean ± SEM] vehicle 1.000 ± 0.05, \(E2\) 0.8330 ± 0.05; \(t=0.2522, df=8.00, p=0.0.357, n=5\) per condition; **Figure 3.4E**). Conversely, an increase in SNAP 25 expression was observed in estradiol treated hippocampal slices from males ([mean ± SEM] vehicle 1.000 ± 0.07, \(E2\) 1.275 ± 0.07; \(t=2.679, df=8.00, p=0.0280, n=5\) per condition; **Figure 3.4C**), but not OVX females ([mean ± SEM] vehicle 1.000 ±
 Estradiol increases a subset of excitatory pre-synaptic proteins in a sex dependent manner after 2 hours. A-F, Representative western blots and quantification of male (A-C) and ovx female (D-F) acute hippocampal slices prepared from 10-12 week old mice and treated with vehicle or estradiol (10 nM, 2 hours). Slices were processed for western blotting and probed for synapsin1, SV2A and snap 25 and normalised to housekeeper, β-actin. Estradiol changed pre-synaptic protein expression levels in a sexually dimorphic manner. Estradiol did not change synapsin1 expression levels in the male (A) or ovx female (D) hippocampus. SV2A expression levels were decreased in ovx females (E) but not males (B) within the same time-frame following estradiol treatment. Estradiol however, increased snap 25 expression levels in the male (C) but not ovx female (F) hippocampus; n=4-6 per condition. Unpaired student’s t-test with Welch’s correction was used with parametric data sets; Mann-Whitney test was used with non-parametric data sets; Error bars represent mean ± SEM; * p = <0.05, ns = not significant.
These data further confirm a sex specific regulation of protein pools found at excitatory synapses by estradiol. Nonetheless, it illustrates that estradiol is able to differentially alter pre-synaptic proteins suggesting that changes at pre-synaptic terminals may contribute to or be a consequence of estradiol-mediated synaptic plasticity in the hippocampus.

Sex specific expression difference is observed in specific pre-synaptic inhibitory proteins

In line with the changes estradiol induces in the expression of post-synaptic inhibitory proteins, it could be reasoned that estradiol may differentially alter critical proteins present pre-synaptically in inhibitory neurons. To this end, the expression profile of GAD-65/67 and vesicular GABA transporter (VGAT), both markers of inhibitory interneurons, was assessed in DMSO or estradiol treated (2 hours) hippocampal slices from males and OVX females. Estradiol significantly increased GAD-65/67 expression in the male ([median] vehicle 0.9918, E2 1.360; u=0, p=0.0286, n=4 per condition; Figure 3.5A), but not in the OVX female ([median] vehicle 0.9998, E2 0.9218; u=4, p=0.3429, n=4 per condition; Figure 3.5C) hippocampus. Interestingly, estradiol did not alter the expression of VGAT in males ([mean ± SEM] vehicle 1.000 ± 0.06, E2 1.085 ± 0.06; t=1.026, df=8.00, p=0.3351, n=5 per condition; Figure 3.5B) but significantly reduced its expression in OVX females ([mean ± SEM] vehicle 1.000 ± 0.04, E2 0.7589 ± 0.04; t=4.0680, df=8.00, p=0.0036, n=5 per condition; Figure 3.5D).

Taken together, these data demonstrate a sexually dimorphic regulation of key inhibitory pre-synaptic proteins by estradiol. Increased GAD-65/67 expression was observed solely in the male hippocampus, suggesting that estradiol may be increasing GABA synthesis, through an unknown mechanism, which ultimately may contribute to estradiol-mediated hippocampal synaptic plasticity. Further, decreased VGAT expression found in the OVX female hippocampus may be indicative of a different mechanism estradiol may employ, to regulate hippocampal synaptic plasticity. This could indicate a consequential event in response to estradiol’s reorganisation of inhibitory post-synaptic sites.
Figure 3.5. Key inhibitory synaptic proteins are differentially regulated by acute estradiol treatment. A-D, Representative western blots and quantification of male (A+B) and ovx female (C+D) acute hippocampal slices prepared from 10-12 week old mice and treated with vehicle or estradiol (10 nM, 2 hours). Slices were processed for western blotting and probed for GAD-65/67 and VGAT and normalised to housekeeper, β-actin. GAD-65/67 expression levels were increased in the male (A) but not ovx female (C) hippocampus in response to 2 hour estradiol treatment. Estradiol had no effect on VGAT expression levels in males (B) but decreased expression levels in ovx female (D) within the same time-frame; n=4-6 per condition. Unpaired student’s t-test with Welch’s correction was used with parametric data sets; Mann-Whitney test was used with non-parametric data sets; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, ns = not significant.
DISCUSSION

Summary of results

This chapter has provided data on the effect of acute estradiol treatment on the expression of key excitatory and inhibitory synaptic proteins in male and OVX female mouse hippocampus (see Table 3.1 for a summary). A subset of post-synaptic proteins were observed to be significantly increased in both male and OVX female hippocampus although, estradiol had no effect on some proteins. Additionally, sex-specific increases and decreases in expression were found in pre-synaptic proteins. Collectively, acute estradiol treatment induced a sex-specific expression change of an array of proteins prominent at both excitatory and inhibitory synapses. Consequently, estradiol may influence both excitatory and inhibitory neurotransmission to regulate hippocampal synaptic plasticity in a sexually dimorphic manner.

Estradiol mediates synaptic plasticity predominantly through post-synaptic neurotransmission in the hippocampus

Estradiol modulates several neuronal processes which may contribute to regulating synaptic plasticity, including increased spinogenesis, increased synaptogenesis, potentiated synaptic transmission and enhanced LTP. Some of these synaptic events have been reported to occur within 2 hour estradiol treatment. For example, Liu et al. (2008) reported that ERβ potentiated CA3-CA1 LTP after 2 hours in female mouse hippocampal slices. The current study found increased expression of key synapse-associated post-synaptic proteins such as, PSD-95 and AMPAR and NMDAR subunits. The increase in PSD-95 and AMPARs are consistent with previous reports (Liu et al., 2008). This suggests that estradiol’s ability to enhance LTP is driven in part by an increase in the expression of key post-synaptic proteins. However, whether this protein increase is occurring at the synapses is unknown. Interestingly, Lui et al. (2008) did not observe increased GluN1 or GluN2B expression in either sex, although increases in GluN1 cluster size has been reported before in response to 24 hour estradiol treatment in primary hippocampal neurons (Jelks et al., 2007). In the current
study, estradiol induced a sex-specific increase in GluN1 in the OVX female, but not male, hippocampus. Differences in estradiol administration (*in vivo* vs. *in vitro*) and treatment time can be accounted for the dissimilarities in Lui et al.’s (2008) results with the current study. Nevertheless, the results from the current study suggest that estradiol increases key proteins typically localised to excitatory synapses thereby, promoting a change in the excitatory synaptic proteome.

PSD-95 and GluN1 are thought to be critical for normal synaptic functioning. Enrichment of these proteins at dendritic spines is seen following synaptic potentiation (Bosch and Hayashi, 2012). In addition, a complex of PSD-95 and GluN1 are thought to be the first proteins to arrive at nascent spines, and are required for synaptogenesis to occur (Chubykin et al., 2007; Gerrow et al., 2006). Therefore, the increase in PSD-95 and GluN1 expression reported here are consistent with previous reports that estradiol increases spinogenesis and synaptogenesis. Work from our lab has shown an increase in the synaptic localisation of PSD-95 and GluN1 within 30 minutes (Sellers et al., 2015b). Critically, this is thought to be achieved without an increase in the expression of these proteins within this time-frame. Thus, a subsequent (after 2 hours) increase in the expression of these proteins may replenish the protein previously trafficked to synapses thereby, providing the neuron with the availability of proteins to either newly formed or potentiated synapses.

Estradiol also increased the expression of other NMDAR subunits implicated in LTP. Multiple studies have reported that GluN2B-containing NMDARs are critical for estradiol-induced enhancement in LTP (Smith and McMahon, 2005, 2006; Smith et al., 2016; Snyder et al., 2011; Vedder et al., 2013). Although, these studies examine these changes following longer estradiol exposures (24-48 hours), they report that GluN2B is a key driver of estradiol-induced synaptic neurotransmission. Interestingly, Potier et al. (2015) recently reported that estradiol transiently decreased GluN2B surface diffusion within 15 minutes whilst, having no effect on GluN2A. Following a longer treatment (24 hour), estradiol increased GluN2B and decreased GluN2A surface diffusion. An impairment of the surface diffusion of both NMDAR subunits inhibited estradiol-mediated increase in dendritic spines suggesting this reorganisation of NMDARs may contribute to estradiol-mediated synaptic plasticity. The current study
demonstrated increases in both total GluN2A and total GluN2B expression. Thus, GluN2A and GluN2B may be working in concert to mediate changes in synaptic plasticity. Potier et al. (2015) proposed a model whereby GluN2B may drive the strengthening of synapses via LTP whereas, the increase in GluN2A may play a role in maintaining these connections. This cannot be determined biochemically within the scope of the current study but it points to an important role for both in mediating estradiol-mediated synaptic plasticity.

The present results also uncovered a unique influence on inhibitory synaptic proteins. Estradiol did not change Nlgn2 expression in either male or OVX female but, a sex-specific increase was seen in gephyrin and the α1 subunit of GABA\(\alpha\)R in the male hippocampus. These results seemingly contradict the findings from our collaborators, who found estradiol destabilised inhibitory synapses (Mukherjee et al., 2017). The authors found decreased α1, α2 and γ2–containing GABA\(\alpha\)Rs and gephyrin synaptic clustering in primary cortical neurons after 2 hour estradiol treatment. However, they had solely measured synaptic, but not total, protein levels whereas, the current study determined changes in total protein levels. Additionally, these effects were observed in mixed sex cortical cultures whereas, hippocampal slices from both males and OVX females were employed in the current study. Mukherjee et al. (2017) also found that estradiol (2 hours) reduced mIPSCs and sIPSCs amplitude in CA1 hippocampus in male slices. In support, a reduction in IPSC transmission has also been previously observed in CA1 hippocampus in OVX female (Tabatadze et al., 2015). However, the results from the current study are derived from measuring total protein levels from the whole hippocampus and so it does not have the same resolution to measure functional changes as the electrophysiology studies benefits from.

Neural information is orchestrated by both excitatory and inhibitory synapses and thus, an appropriate balance must be maintained by each (Levinson and El-Husseini, 2005). Studies illustrate that it is the expression of either Nlgn2 and Nlgn1 that give synapses their inhibitory or excitatory identity (Chubykin et al., 2007; Levinson and El-Husseini, 2005). Notably, Nlgn2 contributes to increases in inhibitory synapses (Chubykin et al., 2007) but its expression was not changed within this study. What was not tested, but would be interesting to determine is whether Nlgn1 expression is also increased within this time-frame. Work from our
lab has previously shown that Nlgn1 expression, and co-localisation with PSD-95 is increased in vitro (Sellers et al. 2015b). In line with increased PSD-95 and AMPAR/NMDAR subtypes, estradiol could increase Nlgn1 within 2 hours. This would suggest estradiol may increase excitatory synapses, whilst having no effect on inhibitory synapses. It has been proposed that estradiol may initially suppress inhibitory neurotransmission which, could lead to regulation of excitatory neurotransmission (Rudick and Woolley, 2001). Yet, an important characteristic of the changes at excitatory and inhibitory synapses observed within this study is that these changes are occurring at the same time. Previous reports of changes found at excitatory (Sellers et al., 2015a; Srivastava et al., 2008) and inhibitory (Mukherjee et al., 2017) synapses suggest a shift towards the excitatory side of the excitatory/inhibitory (E/I) balance. The present results show differential changes in both excitatory and inhibitory proteins. Changes to either can affect the ratio between these two types of input and subsequently, lead to a change in the E/I balance. Critically, disruptions to the E/I balance has been proposed to be implicated in psychiatric disorders such as autism spectrum disorders (Rubenstein and Merzenich, 2003) and schizophrenia (Gao and Penzes, 2015).

**Contribution of estradiol-mediated pre-synaptic neurotransmission**

Within the current study, estradiol also mediated sex-specific changes in pre-synaptic proteins present at both excitatory and inhibitory synapses. Estradiol increased pre-synaptic proteins in the males (GAD-65/67, SNAP 25) in concert with decreased pre-synaptic proteins in the OVX females (VGAT, SV2A) after 2 hour treatment. There were several proteins that did not change such as synapsin 1, SV2A and VGAT in males, and SNAP 25 and GAD-65/67 in OVX females. Nevertheless, estradiol also shifts towards the excitatory side of the E/I balance pre-synaptically. It is unclear whether this is contributing to, or is a consequence, of sex-specific estradiol-mediated changes in the hippocampus.

Increases in specific pre-synaptic proteins are consistent with previous reports. A number of studies found increases following a 24 or 48 hour estradiol treatment (Brake et al., 2001; Jelks et al., 2007; Rune et al., 2002). These time-frames are
much longer than what is of interest in this present study and could point to a consequence of estradiol-induced synaptic neurotransmission. In that, estradiol may rapidly increase a subset of post-synaptic proteins to mediate hippocampal plasticity, and in turn may consequently increase pre-synaptic proteins. Thus, the changes seen in this study could be a transient change. Previously, increased SNAP 25 mRNA, within 1 hour, and protein expression, at 4 hours, was observed in OVX female mouse (Pechenino and Frick, 2009). However, this increased protein expression was not evident at 3 hours. This suggests that estradiol-mediated increase in mRNA levels manifest into increased protein expression 2 hours later. This could explain why changes in SNAP 25 expression was only observed in males, but not OVX females, within the current study. A possibility may be that SNAP 25 expression may not be affected at 2 hours. Other proteins were also changed in a sexually dimorphic manner such as GAD-65/67, VGAT, and SV2A. The expression profile of these proteins could be also changed within a different time-frame to this study. Nevertheless, the sexual dimorphism in the change in protein expression in response to estradiol indicates that estradiol may be regulating the components of the pre-synaptic architecture in a sex dependent manner. This would in turn suggest that males and OVX females may use distinct signalling mechanisms to modulate estradiol-mediated changes.

A sex independent expression change found within this study was that Synapsin 1 was not changed in either sex following 2 hour estradiol treatment. A similar observation was made by Liu et al. (2008) in synaptophysin expression following 4 hour estradiol treatment. Except that following a region specific investigation, synaptophysin expression was found to be changed specifically in pyramidal and radial cell layer of hippocampal CA1. Thus, any region-specific changes in protein expression cannot be ruled out. A caveat of the current study was that the whole hippocampus was used to determine expression changes and so there is less sensitivity to region specific changes. This could be accountable to several proteins that were not found to be affected by estradiol levels. A number of studies found increases following a 24/48 hour estradiol treatment (Brake et al., 2001; Jelks et al., 2007; Rune et al., 2002). These time-frames are much longer than what is of interest in this present study and could point to a consequence of estradiol-induced synaptic neurotransmission. In that, estradiol may rapidly increase a subset of post-synaptic proteins to mediate hippocampal plasticity,
and in turn may consequently increase pre-synaptic proteins. Thus, the changes seen in this study could be a transient change.

Changes in the pre-synaptic proteome suggest that estradiol may be modulating the pre-synaptic architecture. Whether this influences, or is a consequence of, changes it mediates post-synaptically is not clear. Estradiol-mediated increase in glutamate probability release has previously been implicated to contribute to estradiol-induced EPSCs (Smejkalova and Woolley, 2010). This was observed at 30 minutes. The short time-frame within which this occurs may indicate that estradiol could be modulating pre-synaptic machinery prior to mediating post-synaptic changes. Thereby, the changes seen following 2 hour estradiol treatment could be a secondary effect. Estradiol could be modulating changes at pre-synaptic terminals upon application and subsequently the proteomic signature seen at 2 hours in the current study could be the resultant effect. Although, a higher concentration (100 nM) of estradiol was employed compared to the present study (10 nM), the presynaptic protein signature seen may be different at an earlier time-point. Additionally, GAD-65/67 protein (Murphy et al., 1998) and mRNA (McCarthy et al., 1995) expression changes have previously been reported in response to estradiol. This suggests that estradiol may be mediating changes at inhibitory neurons, in addition to inhibitory synapses within this time-frame. Although, this is not the scope of this study, it was of interest to see whether estradiol effects proteins present in inhibitory neurons within this time-frame.

Estradiol induces expression changes in synaptic proteins in a sexually dimorphic manner

One of the biggest caveats in estradiol research is that majority of the studies investigate and report findings on a single sex. Sex differences between males and females are found in hippocampal morphology, physiology and function (Choleris et al., 2018). There is also evidence that estradiol can be produced in both the male and female hippocampus de novo (Hojo et al., 2004; Prange-Kiel et al., 2003). Nonetheless, recently studies into the effect of estradiol in the hippocampus have been taking sex into consideration and have reported the
effect of latent differences, within which the outcome may be the same but the underlying molecular mechanism can be different (Oberlander and Woolley, 2016). The sexually dimorphic regulation of the proteins present in both excitatory and inhibitory synapses observed in this study highlight that estradiol may be regulating hippocampal synaptic plasticity independently of sex. For example, different ERs have recently been found to mediate estradiol-induced excitatory synaptic neurotransmission in the male and OVX female hippocampus (Oberlander and Woolley, 2016). In another case, systemic inhibition of aromatase in male, female and OVX female mouse hippocampus impaired LTP to different degrees in both sexes after 1 day (Vierk et al., 2012); these observations were coupled with spine loss in female and OVX female but not in males. Thereby, considering the data posits sex-specific changes in estradiol-induced protein composition at both types of synapses it highlights the need to incorporate both sexes in future research investigating estradiol-mediated hippocampal synaptic plasticity. This would also give us insights on sex-specific assessments on therapeutic actions of estradiol in the hippocampus.

Conclusions

This chapter demonstrated that estradiol induced a sex-specific change at synapses by regulating the expression of key proteins. Estradiol had different effects on the proteome of both excitatory and inhibitory synapses in each sex. Importantly, it highlights that estradiol increases key excitatory post-synaptic proteins in the hippocampus of both sexes. This is also accompanied by increases in inhibitory proteins GABA\(_\alpha1\)R and gephyrin in males but not in OVX females. Thus, estradiol manipulates a shift towards the excitatory side of the excitatory/inhibitory (E/I) balance. This was also observed pre-synaptically. Therefore, both excitatory and inhibitory proteins are affecting the E/I balance. These data raises multiple questions: 1. whether the consistent increase seen in excitatory post-synaptic proteins in the present study are being mediated through the same ER; 2. whether it is indeed the same receptor but different signalling pathways; and 3. whether estradiol is causing a similar cellular distribution of these proteins. As a first of its kind study, it is informative on the effect estradiol
is exerting on key proteins found at both excitatory and inhibitory synapses, if at all, and whether the output, protein expression, is the same in males and females.

Given the relatively short time-frame within which estradiol increases specific protein expression suggests a non-genomic mechanism of action. Thus, estradiol may be engaging with locally available translational machinery and locally translating specific proteins to mediate these effects on synaptic neurotransmission in the hippocampus. To address whether estradiol is indeed engaging translational machinery, Chapter 4 will focus on determining whether estradiol can drive a change in protein translation within this time-frame.
Table 3.1: Summary of synaptic protein expression change in male and OVX female acute hippocampal slices following a 2 hour estradiol treatment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Synapse identity</th>
<th>Synaptic location</th>
<th>Estradiol-mediated expression change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Excitatory</td>
<td>Postsynaptic</td>
<td>↑</td>
</tr>
<tr>
<td>GluA1</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>GluN1</td>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>GluN2A</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>GluN2B</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>gephrin</td>
<td>Inhibitory</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Nlgn2</td>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>GABAα1</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Synapsin1</td>
<td>Excitatory</td>
<td>Presynaptic</td>
<td>No change</td>
</tr>
<tr>
<td>SV2A</td>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Snap 25*</td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>GAD-65/67</td>
<td>Inhibitory</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>VGAT</td>
<td></td>
<td></td>
<td>No change</td>
</tr>
</tbody>
</table>
CHAPTER 4

ESTROGEN INCREASES LOCAL PROTEIN SYNTHESIS IN A SEXUALLY DIMORPHIC MANNER

SUMMARY

This chapter has focused on determining whether the estradiol-mediated increase in synaptic protein expression levels could be attributable to local protein synthesis. Previous reports have suggested that estradiol can regulate local protein synthesis, but this has not been specifically tested. Employing acute hippocampal slice preparations from both male and OVX female mice, and rat primary hippocampal neurons, this chapter aimed to investigate whether estradiol increased protein synthesis following a 2 hour estradiol treatment. Two independent protein synthesis visualisation assays, SUnSET and FUNCAT, were employed to investigate this. The results revealed an increase in protein synthesis, independently of gene transcription in both sexes. The data further demonstrated that estradiol employed different signalling pathways in male and OVX female rodents to mediate this. Interestingly, newly synthesised proteins could be detected along dendrites and specifically in larger dendritic spines.

This is the first study to illustrate that estradiol rapidly enhances protein synthesis specifically at dendritic and synaptic regions. This demonstrates a novel mechanism by which estradiol may modulate hippocampal synaptic plasticity. Critically, it reiterates the need to consider sex as a variable during estradiol research as the signalling pathways estradiol may employ to mediate different aspects of synaptic plasticity may not be synonymous in both sexes.
INTRODUCTION

Local protein synthesis is deemed a critical component of long-lasing changes in synaptic plasticity, neuronal circuitry and cognition (Costa-Mattioli et al., 2009; Liu-Yesucevitz et al., 2011). It has been demonstrated to occur in dendrites (Steward and Schuman, 2001), axons (Lin and Holt, 2007) and astrocytes (e.g. Sakers et al. 2017). Dendritic synthesis of proteins can rapidly provide synapses with essential proteins circumventing the communication required between the nucleus and synapse to coordinate transcription and subsequent transport of proteins. In neurons, translational machinery such as ribosomes and translation factors are found along dendrites and in dendritic spines in addition, to their presence in the soma (Steward and Schuman, 2001). An abundance of mRNA for synaptic proteins are also present along dendrites (Cajigas et al., 2012). Indeed, local protein synthesis can occur within minutes if the target mRNA is present at the site of translation (Steward and Schuman, 2001). Collectively, this alludes to the notion that both the translational machinery and substrates required for protein synthesis is present at dendrites. This local control of protein synthesis indicates that new proteins can be synthesised within the vicinity of synapses (Holt and Schuman, 2013), bypassing gene transcription and thus, mediate a local control of the synaptic proteome (Rangaraju et al., 2017). Thereby, the local synthesis of proteins provides a mechanism to control synaptic changes independently and may contribute to underlying long-lasting forms of synaptic plasticity (Aakalu et al., 2001).

Indeed, local protein synthesis has been implicated as a critical component of various forms of long-lasting hippocampal synaptic plasticity (Klann et al., 2004). Evidence posits that new protein synthesis is required for the maintenance of L-LTP (Cracco et al., 2005; Kang and Schuman, 1996; Nguyen et al., 1994; Ostroff et al., 2018) and mGluR-dependent LTD (Graber et al., 2013; Huber et al., 2000) in the hippocampus. A number of neuronal events that are independent of new protein synthesis are triggered following the initial stimulation of both phenomena, such as modifications to spine density and morphology and exo/endocytosis of ionotropic glutamate receptors (iGluRs), such as AMPARs and NMDARs. Newly synthesised proteins maintain and stabilise these phenomena by regulating the trafficking of iGluRs (Pfeiffer and Huber, 2006). Some parallels in the
reorganisation of the neuronal architecture can be observed in response to estradiol. Estradiol rapidly, within 30 minutes, promotes protein trafficking and spine modifications *in vitro*, which are independent of new protein synthesis (Sellers et al. 2015b; Srivastava et al. 2008). Whether the stabilisation of these neuronal events require protein synthesis is yet to be determined (Sellers et al. 2015a). Nevertheless, following this time-frame, estradiol increases protein expression in a relatively short time-scale suggesting a non-genomic mechanism of action, such as local protein synthesis. In support, estradiol has previously been demonstrated to increase dendritic mRNA translation of CAMKIIα over 1 hour in primary hippocampal neurons (Sarkar et al., 2010). However, the mechanisms linking estrogenic signalling with local protein synthesis are currently unknown.

A signalling pathway proposed to have a pivotal role in estradiol-induced local protein synthesis is the mTOR kinase signalling pathway. In support, countless studies have illustrated that estradiol rapidly phosphorylates mTOR in the hippocampus within 5 minutes *in vivo* (Fortress et al., 2013) and 15 minutes in the hippocampus and cortex *in vitro* (Briz & Baudry 2014; Sellers et al. 2015b). mTOR is activated by many kinases, one of which is ERK (Winter et al., 2011). Evidence also presents that estradiol rapidly phosphorylates ERK in the hippocampus *in vivo* (Fan et al., 2010; Fernandez et al., 2008; Fortress et al., 2013) and in the hippocampus and cortex *in vitro* (Briz & Baudry 2014; Sellers et al. 2015b); this was specifically seen in the p42 isoform of ERK (Fan et al., 2010). The mTOR pathway has been shown to have a key role in mediating local protein synthesis by phosphorylating two prominent proteins of the translation initiation machinery, S6K and 4EBP1 (Costa-Mattioli et al., 2009; Hoeffer and Klann, 2010; Lipton and Sahin, 2014). The ERK pathway has also been implicated in regulating translation initiation machinery via mTOR (Roux et al., 2007; Tsokas et al., 2007; Winter et al., 2011) and independently of mTOR by activating eukaryotic translation initiation factor 4E (eIF4E) (Banko et al., 2004; Waskiewicz et al., 1999). However, less is known regarding the molecular basis of ERK-mediated protein synthesis. Studies demonstrate that estradiol rapidly modulates the activation of several proteins directly involved in regulating protein translation machinery. Estradiol rapidly phosphorylates: 4EBP1 *in vitro* (Akama and McEwen, 2003; Sarkar et al., 2010) and *in vivo* within 5 minutes (Fortress et al.,
S6K in vivo within 5 minutes (Fortress et al., 2013); and RPS6, a direct downstream target of S6K, in vitro within 10 minutes (Sarkar et al., 2010). The activation of ERK and mTOR are required for estradiol to activate these proteins (Fortress et al., 2013) demonstrating a possible cross-talk between these two kinases. These kinases have also been linked to triggering an increase in synaptic plasticity (Hoeffer and Klann, 2010). Thus, a mechanism is in place to underlie estradiol-induced translation of dendrite-localized mRNA transcripts.

In the previous chapter, estradiol drove an increase in specific synaptic protein expression levels within a short time-frame, indicating the potential involvement of a protein synthesis- but not gene transcription-dependent mechanism. Critically, while estradiol engages translational machinery and activates signalling pathways involved in local protein synthesis, there is no direct evidence that estradiol regulates protein synthesis. This chapter investigated whether estradiol regulates protein synthesis within the same time-frame as it increases synaptic proteins using two established assays of direct visualisation of protein synthesis, SUnSET and FUNCAT. Consequently, given the strong evidence suggesting mTOR and ERK could converge onto protein synthesis machinery, whether either, or both, would be required to mediate estradiol-dependent local protein synthesis remains unclear. Therefore, the role of mTOR in estradiol-mediated effects on protein synthesis was assessed in both male and OVX female hippocampus to determine whether the underlying molecular mechanisms involved in regulating estradiol-mediated protein synthesis were synonymous. Additionally, using primary hippocampal neurons, the sub-cellular location of newly synthesised proteins following estradiol treatment was examined.

**RESULTS**

**Estradiol increases the rate of protein synthesis in hippocampal slices**

To determine whether estradiol regulates protein synthesis directly within the same time-frame as estradiol-induced increases in synaptic protein expression, the rate of protein synthesis in response to estradiol was assessed in the male and OVX female hippocampus. Acute hippocampal slices were prepared from
10-12 week old male and OVX female mice followed by a 2 hour DMSO or estradiol treatment. The SUnSET assay was employed to monitor the effect of estradiol on protein synthesis whereby, 5µg/mL puromycin was added to each condition during the last 30 minutes of treatment. Figure 4.1A illustrates the pharmacological timeline of the experiment. The experimental set up was similar to that of the previous chapter whereby, the hippocampi were divided by hemispheres into two of the different conditions, DMSO and estradiol, per slice (Figure 4.1B). This set up was chosen to favour an internal representation of the effect of estradiol on protein synthesis compared to a vehicle control. The slices were subsequently lysed and puromycin incorporation was measured through western blotting and normalised to house-keeper, β-actin. A significant increase in protein synthesis was observed in slices treated with estradiol compared to DMSO treated slices in both male ([mean ± SEM] vehicle 1.000 ± 0.04, E2 1.378 ± 0.04; t=5.993, df=10.00, p=0.0001, n=6; Figures 4.1C+E) and OVX female ([mean ± SEM] vehicle 1.000 ± 0.04, E2 1.244 ± 0.04; t=4.828, df=8.00, p=0.0013, n=6; Figures 4.1D+F) hippocampus. The SUnSET assay was validated through western blotting and ICC in both DIV 24 primary cortical neurons and male hippocampal slices (Appendix 1A-C). No puromycin incorporation was detected in the absence of puromycin incubation during the pharmacological treatment; the signal is reduced in the presence of anisomycin (Appendix 1A+B). Equally, no signal is detected through ICC in the absence of puromycin incubation (Appendix 1C) inferring the specificity of the antibodies used in the current study.

These data are the first of its kind to show that estradiol acutely increases protein synthesis in the mouse hippocampus and notably, this effect is independent of sex.
Figure 4.1 Estradiol increases protein synthesis in the male and OVX female hippocampus. 

A, Time-line of pharmacological treatments. 

B, Diagram of the experimental set up: acute hippocampal slices were prepared from 10-12 week old mice and treated with vehicle or estradiol within the same animal. 

C+D, Representative western blots of male (C) and OVX female (D) hippocampal slices treated with estradiol (10 nM, 2 hours) and puromycin (SUnSET assay, 5 µg/mL, last 30 minutes), processed for western blotting, probed for puromycin and normalised to housekeeper, β-actin. 

E+F, Quantification of C+D. Estradiol acutely increased the rate of translation in male (E) and OVX female (F) hippocampus; n=5-6 per condition. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; ** p = <0.01, *** p = <0.001.
Estradiol-induced increase in protein synthesis is dependent on a translation mechanism

Although the results corroborated an effect of estradiol on protein synthesis, the data does not differentiate how much of this increase in protein synthesis is a consequence of local protein synthesis. Thus, it was critical to next identify whether this increase is still augmented or absent in the presence of protein synthesis inhibitors; and importantly whether the mechanisms are identical in both sexes. To test this, hippocampal slices were pre-treated with either protein synthesis inhibitor, anisomycin (40µm), or gene transcription inhibitor, actinomycin D (20µm) for 30 minutes. Slices were then treated with DMSO or estradiol for 2 hours followed by the addition of puromycin (Figures 4.2A-B, 4.3A-B). The experimental set up was as previously whereby, all 4 conditions were applied to hippocampal slices from one animal. The slices were divided by hemispheres and sequentially treated with DMSO, estradiol, inhibitor + DMSO, inhibitor + estradiol distributing 3 hippocampal slices per condition. The set up allowed the verification of successful estradiol treatment ensuring that the effects seen in response to respective inhibitors was attributable to the inhibition of that specific mechanism. The slices were subsequently lysed and processed for western blotting and measured for the readout of protein synthesis.

Estradiol-mediated increase in protein synthesis was blocked in the presence of anisomycin in both male (F(3,20)=23.70, p=<0.0001; Figures 4.2C+E) and OVX female (F(3,20)=25.12, p=<0.0001; Figures 4.2D+F) hippocampal slices. A multiple comparisons test with Bonferroni’s correction confirmed an increase in protein synthesis in estradiol treated slices compared to vehicle in males ([mean ± SEM] vehicle 1.000 ± 0.09, E2 1.294 ± 0.09; p=0.0152, n=6) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.10, E2 1.309 ± 0.10; p=0.0249, n=6). However, no difference was observed in slices treated with anisomycin + DMSO compared to anisomycin + estradiol in either male ([mean ± SEM] anisomycin + vehicle 0.700 ± 0.09, anisomycin + E2 0.667 ± 0.09; p>0.9999, n=6) nor OVX female ([mean ± SEM] anisomycin + vehicle 0.608 ± 0.10, anisomycin + E2 0.614 ± 0.10; p>0.9999, n=6). Additionally, anisomycin + estradiol treated slices exhibited
significantly decreased levels of protein synthesis compared to estradiol treated slices in both sexes (p<0.0001 for both).

Conversely, estradiol continued to augment the increase in protein synthesis in the presence of actinomycin D in both male (F(3,16)=15.19, p=<0.0001; Figures 4.3C+E) and OVX female (F(3,20)=31.87, p=<0.0001; Figures 4.3D+F) hippocampal slices. A post hoc Bonferroni’s multiple comparisons test recapitulated a significant difference between DMSO and estradiol treated slices in males ([mean ± SEM] vehicle 1.000 ± 0.12, E2 1.372 ± 0.12; p=0.0399, n=6) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.15, E2 1.459 ± 0.15; p=0.0324, n=6). This increase was conserved when gene transcription was blocked in both male ([mean ± SEM] actinomycin D + vehicle 1.313 ± 0.12, actinomycin D + E2 1.800 ± 0.12; p=0.0053, n=6) and OVX female ([mean ± SEM] actinomycin D + vehicle 1.904 ± 0.15, actinomycin D + E2 2.366 ± 0.15; p=0.0306, n=6) hippocampus. Surprisingly, actinomycin D + DMSO treated slices exhibited an increase in protein synthesis compared to slices treated solely with DMSO in OVX females (p=<0.0001) but not males (p=0.1114).

Taken together, these data suggest that a mechanism that is independent of gene transcription underlies estradiol-mediated increases in protein synthesis, which is synonymous in both male and female mouse hippocampus.
Figure 4.2 Estradiol-mediated increase in protein synthesis is inhibited in the presence of anisomycin. A, Time-line of pharmacological treatments. B, Diagram of the experimental set up: acute hippocampal slices were prepared from 10-12 week old mice and treated with vehicle, with or without anisomycin, or estradiol, with or without anisomycin, within the same animal. C+D, Representative western blots of male (C) and OVX female (D) hippocampal slices pre-treated with anisomycin (40 µM, 30 mins) followed by 2 hour estradiol (10 nM) treatment and puromycin (SUnSET assay, 5 µg/mL, last 30 minutes). Slices were processed for western blotting, probed for puromycin and normalised to housekeeper, β-actin. E+F, Quantification of C+D. Anisomycin inhibited estradiol-mediated increases of the rate of protein synthesis; n=5-6 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, **** p = <0.0001, ns = not significant.
**Figure 4.3** Estradiol increases protein synthesis independent of gene transcription. 

**A,** Time-line of pharmacological treatments. 

**B,** Diagram of the experimental set up: acute hippocampal slices were prepared from 10-12 week old mice and treated with vehicle, with or without actinomycin D, or estradiol, with or without actinomycin D, within the same animal. 

**C+D,** Representative western blots of male (C) and OVX female (D) hippocampal slices pre-treated with actinomycin D (20 µM, 30 mins) followed by 2 hour estradiol (10 nM) treatment and puromycin (SUnSET assay, 5 µg/mL, last 30 minutes). Slices were processed for western blotting, probed for puromycin and normalised to housekeeper, β-actin. 

**E+F,** Quantification of C+D. Estradiol-mediated increases in the rate of protein synthesis were not reduced in the presence of a gene transcription inhibitor; n=5-6 per condition; One-way ANOVA, Bonferroni
mTOR is required for estradiol-dependent increase in protein synthesis in the male, but not OVX female, hippocampus

mTOR kinase signalling has been implicated in mediating local protein synthesis (Hoeffer and Klann, 2010) and countless studies have exhibited estradiol’s ability to rapidly phosphorylate mTOR (Briz and Baudry, 2014; Fortress et al., 2013) in the hippocampus. Specifically, mTOR is still phosphorylated after 1 hour estradiol treatment in acute hippocampal slices (Briz and Baudry, 2014). What is more, mTOR is upstream of a number of protein translation initiation elements. It can be reasoned that this rapid activation of mTOR prior to the induction of protein synthesis observed after 2 hour estradiol exposure may point to a critical role for mTOR in mediating estradiol-dependent increases in protein synthesis. Therefore, hippocampal slices were pre-treated with the mTOR inhibitor, rapamycin (1µM), 30 minutes prior to DMSO or estradiol treatment; puromycin was subsequently added to the slices (Figures 4.4A+B). The slices were lysed and processed for western blotting and measured for puromycin levels and the activation of mTOR. Interestingly, rapamycin inhibited estradiol-mediated increase in protein synthesis in the male (F(3, 20) = 9.616, p=0.0004; Figures 4.4C+E) but not the OVX female (F(3,20)=6.010, p=0.0043; Figures 4.4D+F) hippocampus. A multiple comparisons test with Bonferroni’s correction corroborated increased protein synthesis in estradiol treated slices compared to vehicle in males ([mean ± SEM] vehicle 1.000 ± 0.09, E2 1.330 ± 0.09; p=0.0062, n=6) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.06, E2 1.188 ± 0.06; p=0.0398, n=6). Increases in protein synthesis were inhibited in slices treated with rapamycin + estradiol compared to rapamycin + DMSO treated slices only in males ([mean ± SEM] rapamycin + vehicle 1.025 ± 0.09, rapamycin + E2 0.889 ± 0.09; p=0.7840, n=6) but not OVX females ([mean ± SEM] rapamycin + vehicle 0.9960 ± 0.06, rapamycin + E2 1.181 ± 0.06; p=0.0451, n=6). Specifically, protein synthesis was decreased in slices treated with rapamycin + estradiol in comparison to slices treated with estradiol in males (p=0.0003) however, no difference was observed in OVX females (p>0.9999). Additionally,
protein synthesis levels were found to be significantly higher in estradiol treated slices compared to slices treated with rapamycin + DMSO (male: p=0.0123; OVX female: p=0.0343).

**Figure 4.4** mTOR is required for estradiol-mediated protein synthesis in the male hippocampus. **A**, Time-line of pharmacological treatments. **B**, Diagram of the experimental set up: acute hippocampal slices were prepared from 10-12 week old mice and treated with vehicle, with or without rapamycin, or estradiol, with or without rapamycin, within the same animal. **C+D** Representative western blots of male (C) and OVX female (D) hippocampal slices pre-treated with rapamycin (1 µM, 30 mins) followed by 2 hour estradiol (10 nM) treatment and puromycin (SUnSET assay, 5 µg/mL, last 30 minutes). Slices were processed for western
of C+D. Estradiol-mediated increases in the rate of protein synthesis were inhibited in the male, but not the OVX female, hippocampus; n=5-6 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, *** p = <0.001, ns = not significant.

The observation of a sexual dimorphism in the activation of mTOR being required to induce estradiol-mediated increase in protein synthesis suggests that estradiol may differentially activate mTOR. A similar time-course of mTOR phosphorylation was observed in DIV 25 primary rat cortical neurons (Appendix 2A+B) to that of Briz & Baudry (2014) in male rat hippocampal slices. Primary cortical neurons were prepared from E18 rat embryos and cultured until DIV 25 upon which, the neurons were temporally treated with DMSO or estradiol over 2 hours. Estradiol increased mTOR phosphorylation in a time-dependent manner (F(4,25)=5.875, p=0.0018). A multiple comparison test with Bonferroni’s correction demonstrated a significant increase of mTOR phosphorylation within 30 minutes (p=0.0012), which remained increased at 1 hour (p=0.0128). At 2 hours, mTOR phosphorylation decreased but remained above baseline (p=0.156), although not statistically different from baseline ([mean ± SEM] vehicle 1.00 ± 0.06, 15’ E2 1.055 ± 0.06, 30’ E2 1.263 ± 0.06, 60’ E2 1.204 ± 0.06, 120’ E2 1.147 ± 0.06; n=6 for all). Comparably, this effect was emulated in hippocampal slices from both male (F(3,16)=29.09, p<0.0001; Figures 4.5 A+C) and OVX female after 2 hours of estradiol treatment (F(3,12)=14.64, p=0.0003; Figures 4.5 B+D) hippocampal slices. A post hoc Bonferroni’s multiple comparisons test illustrated no significant difference in mTOR phosphorylation between vehicle and estradiol in either sex ([mean ± SEM] male, vehicle 1.000 ± 0.08, E2 0.8478 ± 0.08, p=0.3793; female: vehicle 1.000 ± 0.07, E2 0.9135 ± 0.07, p>0.9999; n=5, 4 respectively). Concomitantly, estradiol did not increase mTOR phosphorylation in the presence of rapamycin in males nor OVX females ([mean ± SEM] male, rapamycin + vehicle 0.4322 ± 0.08, rapamycin + E2 0.4318 ± 0.08; OVX female rapamycin + vehicle 0.6168 ± 0.07, rapamycin + E2 0.6496 ± 0.07; p>0.9999 for both; n=5, 4 respectively). As expected, mTOR phosphorylation was significantly decreased in rapamycin + DMSO (male, p<0.0001; OVX female,
p=0.0181) and rapamycin + estradiol (male, p<0.0001; OVX female, p=0.0243) treated slices compared to vehicle. This was also true when rapamycin + DMSO (male, p=0.0003; OVX female p=0.0072) and rapamycin + estradiol (male, p=0.0003; OVX female, p=0.0167) treated slices were compared to estradiol treated slices.

**Figure 4.5 Activation of mTOR kinase signalling pathway following 2 hour estradiol treatment.** A+B, Representative western blots of male (A) and OVX female (B) hippocampal slices pre-treated with rapamycin (1 µM, 30 mins) followed by 2 hour estradiol (10 nM) treatment. Slices were processed for western blotting, probed for phospho-mTOR and normalised to total mTOR. C+D, Quantification of A+B. No change in the activation of mTOR was observed compared to total mTOR levels following 2 hour estradiol treatment in the male (C) and OVX female (D) hippocampus. Rapamycin blocked activation of mTOR; n=4-5 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01; *** p = <0.001, **** p = <0.0001, ns = not significant.
Current results and previous reports indicate that estradiol temporally activates mTOR prior to the increase in protein synthesis observed after 2 hours estradiol treatment suggesting that mTOR may be contributing to this increase. However, these data show that mTOR signalling is critical for estradiol to increase protein synthesis in the male rodent hippocampus, but not OVX females. This suggests that although estradiol may be mediating protein synthesis in both sexes, the underlying mechanism specifically, the signalling pathway it utilises, is distinct in each sex.

**Estradiol acutely increases protein translation in crude synaptosomal fractions**

Previous results in this chapter have exhibited rapid increases in protein synthesis following estradiol treatment in both male and OVX female hippocampus. This increase is dependent on a mechanism independent of gene transcription in both sexes though, the biochemical data presented does not allude to the cellular location of this increase. Therefore, it was determined whether estradiol specifically increases protein synthesis at or near synapses.

Primary hippocampal neurons were prepared from E18 rat embryos and cultured until DIV 25-27 upon which, the neurons were treated with DMSO or estradiol for 2 hours; 10µg/mL puromycin was added to each condition 10 minutes prior to lysing. Subsequently, the lysates were fractionated, as detailed in Chapter 2, and puromycin incorporation was measured in the extra-nuclear and crude synaptosomal fractions through western blotting. A significant increase in the rate of protein translation was found in the extra-nuclear fraction ([mean ± SEM] vehicle 1.000 ± 0.10, E2 1.531 ± 0.10; t=3.633, df=4.00, p=0.0221, n=3; Figures 4.6A+C) following estradiol treatment compared to vehicle. Equivalently, estradiol also increased the rate of protein translation specifically in the crude synaptosomal fraction ([mean ± SEM] vehicle 1.000 ± 0.03, E2 1.361 ± 0.03; t=8.861, df=4.00, p=0.0009, n=3; Figures 4.6B+D) compared to vehicle.
The results from the primary neurons mirror the estradiol-dependent increase in protein synthesis observed in hippocampal slices and specifically, infer a spatial location for where this increase is occurring.

**Figure 4.6** Estradiol acutely increases protein synthesis in crude synaptosomal fractions from primary hippocampal neurons. A+B, Representative western blots of DIV 27 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) or vehicle and puromycin (SUnSET assay, 10 µg/mL, last 10 minutes) fractionated upon lysing to separate out the extra-nuclear and crude synaptosomal fractions. Fractions were processed for western blotting, and probed for puromycin and normalised to housekeeper, β-actin. C+D, Quantification of A+B. Rate of translation was increased in estradiol treated
neurons. Estradiol increased the rate of translation in the extra-nuclear (C) and crude synaptosomal (D) fractions; n=3 per condition. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05, *** p = <0.001.

Newly synthesised proteins are found in dendritic spines and along dendrites following 2 hour estradiol treatment

Protein synthesis at dendrites can rapidly target nascent proteins to specific parts of the synaptic architecture in response to neuronal activity (Holt and Schuman, 2013; Steward and Schuman, 2001). This allows a synapse specific control and replenishment of the synaptic proteome. The SUnSET assay demonstrated that estradiol rapidly increases the rate of translation specifically in crude synaptosomal fractions. The assay is an established approach, employed in multiple cell and tissue types (Goodman and Hornberger, 2013), which provides a readout of protein synthesis determined by puromycin incorporation into elongating polypeptide chains. This process however, leads to an early termination of the translation of corresponding mRNAs resulting in truncated peptides tagged with puromycin to be released from the ribosomes. Therefore, SUnSET illustrates how much translation is occurring in response to a treatment but does not inform about the distribution of newly synthesised proteins. A more suitable approach to measure newly synthesised proteins is FUNCAT (detailed in Chapter 2), which drives the incorporation of a ‘methionine surrogate’ into nascent proteins being formed (tom Dieck et al., 2012). Therefore, FUNCAT was employed to determine the spatial localisation of newly synthesised proteins following estradiol treatment.

The FUNCAT reaction was optimised in primary cortical and hippocampal neurons (Appendix 3A-C). The incubation time and concentration for AHA was chosen following the temporal optimisation outlined by Dieterich et al. (2010). DIV 24 primary cortical neurons were incubated with 4mM AHA for 30 and 120 minutes to determine sensitivity for the detection of newly synthesised proteins along neuronal secondary and tertiary dendrites; 120 minutes incubation was favoured by higher AHA intensity compared to 30 minutes (Appendix 3A). In the absence of AHA incubation during pharmacological treatment, tagging with an
alkyne bearing fluorophore Alexa-555 (Alkyne-555) did not bind and fluoresce any signal. Additionally, AHA intensity is decreased in the presence of anisomycin (Appendix 3B). Resonating the findings from Dieterich et al. (2010), newly synthesised proteins could be seen in spine like protrusions in hippocampal neurons (Appendix 3C).

Primary hippocampal neurons were transfected with eGFP at DIV 12 and cultured until DIV 20-21. Neurons were simultaneously treated with AHA (4mM) and either DMSO or estradiol for 2 hours. Following the treatment, neurons were fixed and Alkyne-555 was bound to the AHA via click chemistry. The eGFP was thereafter amplified using a GFP specific antibody and subsequently imaged on a confocal microscope. AHA intensity was crudely measured across approximately 100 µm sections of secondary and tertiary dendrites (as detailed in the Chapter 2, Figure 6); the crude section comprised of the dendritic and dendritic spine regions. AHA intensity was significantly increased in the crude dendritic and spine regions ([median] vehicle 1.063, E2 1.357; u=45, p=0.0439, n=13 for both; Figures 4.7A+B) following a 2 hour estradiol treatment, suggesting that there was an increase in newly synthesised proteins specifically in dendritic and spine regions. When assessed separately, estradiol increased new proteins specifically in dendrites ([mean ± SEM] vehicle 1.000 ± 0.19, E2 2.324 ± 0.51; t=2.432, df=24.00, p=0.0229, n=13 for both; Figures 4.7C) and spines ([median] vehicle 1.021, E2 1.946; u=41, p=0.0225, n=13 for both; Figures 4.7D). These data provide supporting evidence that estradiol-mediated increased protein synthesis results in newly synthesised protein localised along dendrites and within dendritic spines.
Figure 4.7 AHA-tagged newly synthesised proteins are increased along dendrites and within dendritic spines. A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) and AHA (4 mM, 2 hours), tagged with 2 mM Alyne-555 and immunostained for GFP. B, Quantification of A. Estradiol increased synthesis of new proteins in dendritic and spine regions measured crudely. C+D, Quantification of A. Estradiol specifically increased newly synthesised proteins along dendrites (C) and within dendritic spines (D); n=13 per condition. Mann-Whitney test; Error bars represent mean ± SEM; * p < 0.05. Arrowheads denote nascent proteins localised at dendrites (yellow) and dendritic spines (white). Scale bar = 10 µm.
New proteins are synthesised independently of gene transcription through the mTOR signalling pathway in dendritic regions in response to estradiol.

To compliment the biochemical data presented previously, it was determined whether estradiol increased new proteins within dendrites and dendritic spines independently of gene transcription. Additionally, whether mTOR was required for this process was also considered. As above, transfected (eGFP) DIV 20-21 primary hippocampal neurons were pre-treated with either 40µM anisomycin, 20µM actinomycin D or 10nM rapamycin 30 minutes prior to the addition of DMSO + AHA or estradiol + AHA for 2 hours. Neurons were fixed subsequently followed by binding of Alkyne-555 via click chemistry and imaged on a confocal microscope. Estradiol-mediated increase in newly synthesised proteins was blocked in the presence of anisomycin ($H_{(3)}=19.96$, $p=0.0002$; Figures 4.8A+B) but not actinomycin D ($H_{(3)}=21.11$, $p<0.0001$; Figures 4.9A+B). A post hoc Dunn’s multiple comparisons test identified a significant difference in AHA intensity between neurons treated with DMSO or estradiol ([mean rank] vehicle 28.38, E2 44.00; $p=0.0474$, n=16, 17 respectively), whilst there was no difference between neurons treated with anisomycin + DMSO and anisomycin + estradiol ([mean rank] anisomycin + vehicle 19.73, anisomycin + E2 20.86; $p>0.9999$, n=11, 14 respectively). Consequently, AHA intensity was significantly higher in estradiol treated neurons compared to anisomycin + DMSO ($p=0.0012$) and anisomycin + estradiol ($p=0.0009$). Comparably, neurons treated with actinomycin D + estradiol exhibited higher AHA intensity than neurons treated with actinomycin D + DMSO ([mean rank] actinomycin D + vehicle 15.79, actinomycin D + E2 33.57; $p=0.0482$, n=14 for both) mirrored by neurons treated with solely estradiol compared to DMSO ([mean rank] vehicle 27.63, E2 44.59; $p=0.0365$, n=16, 17 respectively). No difference in AHA intensity was observed between neurons treated with estradiol and actinomycin D + estradiol treated neurons ($p=0.5132$). Thus, estradiol continued to increase the synthesis of new proteins in the presence of a gene transcription inhibitor.

Rapamycin inhibited estradiol from increasing newly synthesised proteins within the dendritic and dendritic spine area ($H_{(3)}=15.75$, $p=0.0013$; Figures 4.10A+B). A multiple comparisons test with Dunn’s correction confirmed increased AHA...
intensity in estradiol treated neurons compared to DMSO ([mean rank] vehicle 25.69, E2 42.53; p=0.0292, n=16, 17 respectively). Whilst no difference in AHA intensity was detected in the neurons treated with rapamycin + estradiol compared to rapamycin + DMSO neurons ([mean rank] rapamycin + vehicle 29.86, rapamycin + E2 18.17; p=0.5016, n=14, 12 respectively). Consequently, a decrease in AHA intensity was observed in neurons treated with rapamycin + estradiol compared to those treated solely with estradiol (p=0.0010) suggesting a critical role for mTOR in mediating estradiol-mediated increase in new proteins in dendritic and spine regions.

Taken together, these data show that estradiol uses a translation-dependent mechanism employing the mTOR signalling pathway to increase the synthesis of new proteins specifically in dendritic and spine regions. These data recapitulated the previous findings from hippocampal slices reported earlier in the chapter.
Figure 4.8 Anisomycin inhibits the increase of nascent proteins along dendrites and within dendritic spines in estradiol treated neurons. A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons pre-treated with anisomycin (40 µM, 30 mins) followed by estradiol (10 nM, 2 hours) and AHA (4 mM, 2 hours) treatments. Neurons were thereafter, tagged with 2 mM Alyne-555 and immunostained for GFP. B, Quantification of A. Estradiol increased newly synthesised proteins in dendritic and spine regions, which was blocked by anisomycin; n=12-17 per condition. Kruskal-Wallis test, Dunn’s corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, *** p = <0.001, ns = not significant. Arrowheads denote nascent proteins localised at dendrites (yellow) and dendritic spines (white) and lack thereof (red). Scale bar = 10 µm. A = anisomycin abbreviated.
Figure 4.9 Estradiol treated neurons exhibit increased nascent proteins in dendritic and spine regions in the presence of actinomycin D. A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons pre-treated with actinomycin D (20 µM, 30 mins) followed by estradiol (10 nM, 2 hours) and AHA (4 mM, 2 hours) treatments. Neurons are thereafter, tagged with 2 mM Alyne-555 and immunostained for GFP. B, Quantification of A. Estradiol increased newly synthesised proteins in dendritic and spine regions and continued to do so in the presence of actinomycin D; n=14-17 per condition. Kruskal-Wallis test, Dunn’s corrected; Error bars represent mean ± SEM; * p = <0.05, **** p = <0.0001, ns = not significant. Arrowheads denote nascent proteins localised at dendrites (yellow) and dendritic spines (white). Scale bar = 10 µm. AD = actinomycin D abbreviated.
**Figure 4.10** *mTOR is required for estradiol to increase new proteins along dendrites and spines.* 
A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons pre-treated with rapamycin (1 µM, 30 mins) followed by estradiol (10 nM, 2 hours) and AHA (4 mM, 2 hours) treatments. Neurons are thereafter, tagged with 2 mM Alyne-555 and immunostained for GFP. 
B, Quantification of A. mTOR blocked estradiol-mediated increases of newly synthesised proteins at dendritic and spine regions; n=12-17 per condition. Kruskal-Wallis test, Dunn’s corrected; Error bars represent mean ± SEM; * p = <0.05, ** = p <0.01, ns = not significant. Arrowheads denote nascent proteins localised at dendrites (yellow) and dendritic spines (white) and lack thereof (red). Scale bar = 10 µm. R = rapamycin abbreviated.
Estradiol induces spinogenesis within the same time-frame

Dendritic spines can be arbitrarily classified by their morphology (Hering and Sheng, 2001) as: ‘filopodia-like’; thin, ‘lollipop like’; stubby; or ‘mushroom-like’ (Berry and Nedivi, 2017; Hering and Sheng, 2001; Srivastava et al., 2011). As stated in the previous chapter, many studies have reported spinogenesis following 30 minute estradiol treatment (Inagaki et al., 2012; MacLusky et al., 2005; Phan et al., 2012; Tuscher et al., 2016). Estradiol-mediated increases in spine density have also been observed in male (Jacome et al., 2016; Mukai et al., 2007) and OVX female mouse hippocampus (Inagaki et al. 2012; Phan et al. 2015; Tuscher et al. 2016) following 2 hour estradiol treatment. Particularly, Mukai et al. (2007) saw an increase in thin and filopodia-like spines with a decrease in average spine diameter in hippocampal slices. As spine morphology is thought to be associated with synaptic function, the ability of estradiol to modulate spine density and morphology was assessed within the current study. Principally, the class of dendritic spines that contain nascent proteins was assessed following estradiol treatment.

Transfected (eGFP) DIV 20-21 primary hippocampal neurons treated with either DMSO + AHA or estradiol + AHA for 2 hours were fixed followed by Alkyne-555 binding; the neurons were thereafter, immunostained and imaged on a confocal microscope. Consistent with previous findings, estradiol significantly increased spine density after 2 hours (spine density per 10 µm: [mean ± SEM] vehicle 2.376 ± 0.30, E2 3.357 ± 0.37; t=2.071, df=24.00, p=0.0493, n=13 for both; Figures 4.11A+B). Analysis of dendritic spine morphology revealed that treatment with estradiol resulted in a larger spine area (spine area (µm²): [mean ± SEM] vehicle 0.4120 ± 0.01, E2 0.4732 ± 0.02; t=2.402, df=19.54, p=0.0264, n=13 for both; Figures 4.11A+C). A closer analysis of the morphological changes induced by estradiol illustrated an increase in spines with an area larger than 0.6 µm² where at control levels there were more smaller spines, between 0.2-0.4 µm² (Figures 4.11A+D). Thus, estradiol induced an increase in larger spines after a 2 hour treatment.
Figure 4.11 Estradiol increases spine number and area within 2 hours. A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) and immunostained for GFP. B-D, Quantification of A. Estradiol acutely increased dendritic spine density (B) and spine area (C) after 2 hours. Histogram of spine area showed that estradiol increased spines with a larger area (D); n=13 per condition. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05. Yellow dashed box denotes section of dendrite displayed in inset. Scale bar = 50 μm; 10 μm (inset).
Consequently, the number and morphological profile of spines that contained AHA-tagged newly synthesised proteins were evaluated. No significant difference in AHA intensity was observed in AHA positive spines in estradiol treated neurons (spine density per 10 µm: [mean ± SEM] vehicle 0.9399* ± 0.16, E2 1.290 ± 0.25; t=1.166, df=20.23, p=0.2572, n=12, 13 respectively; Figures 4.12A+B; *one cell data removed following Grubb’s test for outliers). However, analysis of spine morphology illustrated an increase of larger spines containing AHA in response to estradiol treatment (spine area (µm²): [mean ± SEM] vehicle 0.4035 ± 0.02, E2 0.4869 ± 0.03; t=2.214, df=17.99, p=0.0400, n=13 for both; Figures 4.12A+C). Categorising spines that were positive for AHA by morphology displayed a shift in the spine area that contained AHA. Estradiol increased AHA intensity in larger spines, with an area larger than 0.8µm², where more AHA could be observed in smaller spines, areas smaller than 0.8µm², at control levels (Figures 4.12A+D) confirming that there is an increased level of new proteins in bigger spines in estradiol treated neurons compared to vehicle.

These data show that estradiol increases spine number and size after 2 hours. Interestingly, it is the larger spines that contain increased levels of AHA-tagged newly synthesised proteins in estradiol treated neurons in response to estradiol suggesting that newly synthesised proteins are predominantly localised in larger spines following 2 hour estradiol treatment.
Figure 4.12 New proteins are localised to larger dendritic spines in estradiol treated neurons. A, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) and AHA (4 mM, 2 hours) treatments. Neurons are thereafter tagged with 2 mM Alyne-555 and immunostained for GFP. B-D, Quantification of A. Estradiol treated neurons did not show increased AHA-positive spines (B) however, increased AHA-tagged newly synthesised proteins were localised to larger spines compared to vehicle (C). Histogram of spine area of AHA positive spines showed increased AHA expression in larger spines (D); n=13 per condition. Unpaired
student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p < 0.05, ns = not significant. Yellow dashed box denotes section of dendrite displayed in inset. Arrowheads denote nascent proteins localised at dendrites (yellow) and dendritic spines (white). Scale bar = 50 µm; 1 µm (inset).

DISCUSSION

Summary of results

This chapter has demonstrated a novel mechanism by which estradiol may be employing to mediate synaptic plasticity in the hippocampus, specifically through modulating local protein synthesis. Estradiol increased global protein synthesis in both male and OVX female mouse hippocampus within 2 hours, independent of gene transcription. Newly synthesised proteins could specifically be observed along dendrites and in dendritic spines. Critically, the underlying signalling pathways estradiol engaged were different between males and OVX females. The mTOR signalling pathway was found critical for estradiol to mediate an increase in protein synthesis in the male, but not OVX female hippocampus. Together, these data detail how rapid modulation of local protein synthesis by estradiol may result in an increase in synaptic function in males and OVX females underlying the facilitation of cognition offered by estrogens. Importantly, this occurs via distinct signalling mechanisms between sexes.

Estradiol increases protein synthesis, independent of gene transcription, in hippocampal neurons

The results from the current chapter are consistent with the previous notions of a concomitant relationship between estradiol and local protein synthesis. Biochemically, studies have shown rapid activation of protein synthesis initiation machinery, that tightly regulate protein synthesis, in the hippocampus following estradiol treatment; within a time-frame that is consistent with the current study (Akama and McEwen, 2003; Briz and Baudry, 2014; Fortress et al., 2013; Sarkar et al., 2010). Through live-imaging, estradiol has been illustrated to increase dendritic mRNA translation of CAMKIIα, a key protein implicated in LTP and
various forms of memory (Giese and Mizuno, 2013; Miller et al., 2002), in vitro within a similar time-frame (Sarkar et al., 2010). Indeed, this chapter provides confirmation that estradiol rapidly regulates protein synthesis without engaging gene transcription in the hippocampus providing a rationale for the rapid increases of synaptic proteins observed in Chapter 3 and by others (eg. Liu et al. 2008).

By employing two independent assays, estradiol was demonstrated to rapidly increase protein synthesis in vitro both in primary hippocampal neurons and in a more intact system - acute hippocampal slice preparations. A drawback of using SUNSET is that puromycin is incorporated into elongating polypeptide chains. Thus, biochemically employing SUNSET provides invaluable detail on how estradiol influences the rate of protein translation, but not where the newly synthesised proteins localise. By coupling FUNCAT and ICC, the subcellular distribution of newly synthesised proteins was inferred in estradiol-treated neurons. Visualising increases of nascent proteins specifically within subcellular compartments that expresses the machinery and substrates needed for protein synthesis (Cajigas et al., 2012; Holt and Schuman, 2013; Steward and Schuman, 2001) suggests that estradiol may be driving the synthesis of these new proteins along dendrites and in spines within 2 hours to drive changes in hippocampal synaptic plasticity. A recurrent observation from Figures 4.7-4.9 suggests that there may be two populations of neurons within the estradiol treatment condition that encompass AHA-tagged newly synthesised proteins. A reasoning for this could be that only a subset of neurons is responsive to the estradiol treatment. In support, albeit using an in vivo approach, Oberlander and Woolley (2016) reported that only a subset of neurons (~20%) were responsive to estradiol when measuring mEPSCs. Although the authors were applying estradiol at a single neuron level, estradiol is applied to the whole coverslip of neurons within the current experimental set up. Additionally, only a subset of neurons is successfully transfected with eGFP within a coverslip and therefore, the ratio of the number of neurons that have both responded to estradiol and are eGFP positive may vary from biological replicates. Therefore, the results may reflect neurons that have both responded and not responded to the estradiol treatment.
Using hippocampal slices permitted the exploration of how estradiol, if at all, mediates local protein synthesis in both males and females. Moreover, an advantage of using this system to investigate this phenomena is that the integrity of majority of the synaptic connections within the hippocampus is retained (Lein et al., 2011). This was specifically useful for the nature of this current study. Nevertheless, by approaching this hypothesis biochemically using hippocampal slices denoted that the output of the effect of estradiol on local protein synthesis did not solely represent dendritic protein synthesis. Indeed axons (Milner et al., 2001) and astrocytes (Azcoitia et al., 1999; Santagati et al., 1994) also express ERs in addition to post-synaptic compartments in the hippocampus, suggesting that the exogenously applied estradiol could be binding to ERs in neuronal and non-neuronal compartments. Nevertheless, the non-genomic estradiol-mediated increase in protein synthesis observed in slices was recapitulated in primary hippocampal neurons through ICC. Corroborating the findings of rapid dendritic mRNA translation by estradiol (Sarkar et al., 2010), the current results demonstrated increased protein translation in crude synaptosomal fractions and increased nascent proteins along dendrites and within dendritic spines in estradiol treated hippocampal neurons. However, an avenue worth exploring is investigating whether estradiol affects protein synthesis in astrocytes or axons. It is of note, however, that estradiol did not affect the expression of select pre-synaptic proteins examined in Chapter 3, whereas increases were seen in select inhibitory and excitatory post-synaptic proteins, possibly suggesting that estradiol-mediated local protein synthesis is predominately focused on post-synaptic targets in this time-frame.

Distinct signalling mechanisms mediate local protein synthesis between sexes

As observed in the previous chapter, both male and OVX female hippocampus can respond to estradiol in different ways. The results of the current study highlight the significance of considering both sexes during estradiol research and the potential effect of latent differences (Oberlander and Woolley, 2016). Although, estradiol non-genomically increases local protein synthesis in both sexes, the mTOR pathway was found to be required to mediate global protein
synthesis in the male, but not OVX female, hippocampus. mTOR activates local protein synthesis machinery such as 4EBP1, S6K and RPS6 (Costa-Mattioli et al., 2009; Hoeffer and Klann, 2010; Lipton and Sahin, 2014) and has been shown to be required for the establishment of L-LTP (Tang et al., 2002). Inhibition of mTOR prevents estradiol mediated increases in phosphorylation of 4EBP1 and S6K, impairs OR memory (Fortress et al., 2013) and prevents estradiol-induced increases in spine density in CA1 (Tuscher et al. 2016b). This suggests a critical role for mTOR in mediating hippocampal memory and spine density in OVX female rodents, possibly through local protein synthesis. Estradiol-mediated enhancement in memory and dendritic spines have also been observed in male rodents but the underlying cell signalling pathways involved are unknown. Nevertheless, this is the first study to suggest a critical role for mTOR signalling in the male hippocampus.

The current data suggests that estradiol utilises another signalling pathway to mediate protein synthesis in the OVX female hippocampus. The ERK signalling pathway has been reported to be necessary for enhancing CA1 spine density (Tuscher et al. 2016b), OR (Fan et al., 2010; Fortress et al., 2013) and OP (Fortress et al., 2013) memory tasks in response to estradiol in OVX female rodents. Inhibition of ERK prevents estradiol from activating mTOR signalling proteins 4EBP1 and S6K (Fernandez et al., 2008; Fortress et al., 2013; Sarkar et al., 2010) insinuating that ERK also regulates estradiol’s ability to regulate proteins implicated in initiating protein synthesis. Critically, estradiol has been shown to rapidly phosphorylate both mTOR and ERK signalling pathways in male and OVX female hippocampus (Akama & McEwen 2003; Briz & Baudry 2014; Fan et al. 2010; Fernandez et al. 2008; Fortress et al. 2013; Tuscher et al. 2016b) and thus, both pathways are active prior to the increased protein synthesis observed at 2 hours. Nevertheless, the downstream targets they engage are unknown. Critically, despite the dogma that mTOR regulates protein synthesis, it also regulates a number of other cellular processes (Saxton and Sabatini, 2017) thus, mTOR could be mediating estradiol’s effect on another cellular process in the OVX female hippocampus. Alternatively, owing to the system used to investigate this, the effect of mTOR may be too specific to be seen as an overall effect on global protein translation and its action may be more specific. In support, mTOR was critical for estradiol to increase nascent proteins locally in synaptic
regions. Consequently, mTOR may be key in modulating estradiol-mediated protein synthesis in the male hippocampus but may have more specific effects within the female hippocampus. Alternatively, estradiol could be utilising another pathway, such as ERK, to regulate protein synthesis in OVX females.

**Newly synthesised proteins are found in larger dendritic spines**

Increased AHA tagged-newly synthesised proteins were found along dendrites and within dendritic spines of estradiol treated neurons. Recapitulating the biochemical data, estradiol increased newly synthesised proteins independently of gene transcription and through an mTOR dependent manner within the dendritic and spine regions. The biochemical data confirmed that estradiol increases the rate of translation in the rodent hippocampus but did not indicate where newly synthesised proteins are localised. Thereby, suggesting that estradiol could be engaging local machinery available along dendrites to promote the synthesis of new proteins within the vicinity of spines.

In line with previous findings, estradiol increased dendritic spines density over 2 hours (Jacome et al. 2016; Mukai et al. 2007; Tuscher et al. 2016b). Specifically, larger spines were found to be increased. Conversely, Mukai et al. (2007) found that estradiol increases the density of thinner spines after 2 hours. The differences in observation could be due to the use of different systems; Mukai et al. (2007) assessed spine morphology in hippocampal slice preparations from male rats whereas, the current study used primary hippocampal neurons. Moreover, they also used a lower concentration of estradiol (1 nM). Nevertheless, the current data suggests that estradiol may promote stronger synapses, which is consistent with previous reports of estradiol facilitating glutamatergic synaptic transmission (Oberlander and Woolley, 2016). Although, it is not clear whether pre-existing or estradiol-induced nascent spines are enlarged. Nevertheless, spine enlargement depends on protein synthesis (Tanaka et al., 2008) and the current study shows that it is specifically the larger spines that contain AHA-tagged newly synthesised proteins suggesting that new proteins are travelling to these larger dendritic spines. In support, Sambandan et al. (2017) demonstrated that following activity-dependent stimulation, there is increased protein synthesis
at the dendritic shaft of or within the potentiated dendritic spine. Consistent with this notion, there is some level of specificity in the estradiol-mediated synthesis of proteins being targeted to larger dendritic spines. The identity of these new proteins is unknown however, they could contribute to the stability of the dendritic spines or increasing synaptic transmission of that synapse.

Conclusions

This chapter illustrated that estradiol increases protein synthesis, independently of gene transcription, in both male and OVX female hippocampus. This is dependent on the mTOR signalling pathway in males but estradiol seems to be engaging a different signalling pathway in OVX females. As different signalling pathways seem to be critical in mediating estradiol’s effects on protein synthesis in both sexes, it would be interesting to see which ER is important in enhancing protein synthesis and whether the receptors responsible for enhancing protein synthesis in each sex is synonymous. Furthermore, estradiol specifically increases the density of larger dendritic spines and augmented levels of newly synthesised proteins can be observed in larger dendritic spines suggesting that new proteins may be contributing to the stability to these new dendritic spines. This chapter offers a novel mechanism by which estradiol may be regulating synaptic plasticity in the rodent hippocampus.
CHAPTER 5

ESTROGEN INCREASES SYNAPTIC PSD-95 AND GLUN2B IN A LOCAL PROTEIN SYNTHESIS DEPENDENT MANNER

SUMMARY

The previous chapters have demonstrated that estradiol rapidly increases the expression of key synaptic proteins in both male and OVX female hippocampus, concurrent to increasing protein synthesis independently of gene transcription. This is observed within the same time-frame in both male and OVX female rodent hippocampus. Using primary hippocampal neurons, this chapter aimed to determine whether the estradiol-mediated increase in PSD-95 and GluN2B expression levels observed in Chapter 3, were increased via a local protein synthesis-dependent mechanism. Importantly, it was examined whether males and OVX females both use the same signalling mechanism to regulate candidate protein expression. Furthermore, with the knowledge that there is a high presence of translational machinery and substrates along dendrites and below dendritic spines (Steward and Schuman, 2001), and employing super-resolution imaging, the spatial distribution of translation, through SUnSET-ICC, and ribosomal proteins was determined following 2 hour estradiol treatment.

PSD-95 and GluN2B were both found to be increased independently of gene transcription by estradiol, through distinct signalling pathways in the male and
OVX female hippocampus. Estradiol also increased the presence and size of a candidate ribosomal protein and translation within synaptic regions and along dendrites within the same time-frame.

INTRODUCTION

Subcellular localisation of specific proteins enables local and specialised control of cellular compartments. For example, dendritic mRNAs can be locally translated to provide this localised response to extracellular stimuli. The presence of a rich pool of dendritic mRNAs involved in translation initiation and elongation, and synaptic plasticity (Cajigas et al., 2012; Eberwine et al., 2002; Poon et al., 2006; Zhong et al., 2006), to name a few, have been identified employing various methods. GluN2B mRNA has been observed along dendrites of primary rat hippocampal neurons (Miyashiro et al., 1994) and hippocampal slices (Cajigas et al., 2012) in addition to dendritic mRNA for PSD-95 in hippocampal slices (Cajigas et al., 2012). Therefore, the mRNAs for these candidate proteins are ideally situated to be locally translated through estradiol specifically in the hippocampus.

Both PSD-95 and GluN2B are localised at excitatory synapses implicated to have critical roles at synapses and several studies have shown that estradiol can rapidly interact with these proteins. PSD-95 has been shown to be enriched at PSDs (Sugiyama et al., 2005) with a key role in synapse formation (Washbourne et al., 2002) and activity dependent spine stabilisation through its ability to scaffold and localise a number of synaptic proteins (Chubykin et al., 2007). Our lab has shown estradiol-mediated increases of PSD-95 containing spines and neuroligin-1/PSD-95 complexes within 30 minutes in vitro (Sellers et al. 2015b); neuroligin-1/PSD-95 complexes are thought to be needed for functional synaptic connections (Gerrow et al., 2006). Others have found that estradiol increases PSD-95 expression within 4 hours (Liu et al., 2008). In support of this, it has previously been reported that PSD-95 can be synthesised, independently of gene transcription, in a differentiated neuroblastoma cell line (d-NG108-15) following 6 hour estradiol treatment, identified by $^{[35]}$S]-methionine/cysteine pulse labelling (Akama and McEwen, 2003). Furthermore, GluN2B-containing synaptic NMDARs have been exhibited to be important for LTP (Barria and Malinow, 2005;
Berberich et al., 2007; Tang et al., 1999) but, also shown to be involved in LTD (Bartlett et al., 2007). Activation of GluN2B-containing synaptic NMDARs have been implicated in mediating estradiol-induced increase in LTP magnitude (Smith and McMahon, 2006; Smith et al., 2016; Vedder et al., 2013). Thus, both proteins have been demonstrated to be important in estradiol-mediated regulation of synaptic plasticity in both the hippocampus and cortex.

The machinery that estradiol may require to orchestrate the local translation of PSD-95 and GluN2B can be found along dendrites. In addition to the localisation of their respective mRNAs along dendrites (Cajigas et al., 2012), polyribosomes can also be typically found along dendrites specifically in or below dendritic spines (Steward and Levy, 1982; Steward and Schuman, 2001). Spines can also encompass multiple polyribosomes suggesting multiple pre-existing mRNA transcripts may be being translated within or near a single spine (Ostroff et al., 2018). Polyribosomes have been found to accumulate along dendrites in response increased levels of estrogen during the rat estrous cycle (McCarthy and Milner, 2003). Additionally, both ERα and ERβ have also been localised at dendrites (Sellers et al. 2015b) and estradiol activates signalling kinases along dendrites that can mediate a number of cellular changes (Srivastava, personal communication). Thus, estradiol may be able to influence the machinery that is locally present near spines to engineer the translation of pre-existing mRNA transcripts. This is in line with the data from Chapter 4, whereby an increase in new proteins can be visualised along dendrites and specifically, within larger spines. However, it is unknown where this translation could be occurring. Nevertheless, data from Chapter 4 alludes to increases of the rate of translation in crude synaptosomal fractions.

This chapter firstly tries to decipher whether estradiol-mediated increases in excitatory postsynaptic proteins, PSD-95 and GluN2B, could be dependent on local protein synthesis in both male and OVX female hippocampal slices. Moreover, mTOR has been demonstrated to have a pivotal role in mediating estradiol-dependent protein synthesis in the male hippocampus and so its role in mediating estradiol-dependent increases in both PSD-95 and GluN2B is assessed in both sexes. Finally, the spatial distribution of ribosomal protein S10 (RPS10), member of the smaller 40S ribosomal subunit, was assessed within 2
hours to determine the acute effects of estradiol on ribosomes at synapses. Coupled with SUnSET-ICC and super resolution imaging, the spatial distribution of where estradiol could be facilitating translation was investigated.

**RESULTS**

**Estradiol increases expression of PSD-95 and GluN2B in a translation dependent manner**

Chapter 3 reported an increase in PSD-95 and GluN2B expression levels in both male and female hippocampus following 2 hour estradiol treatment. As alluded previously, the short time-frame within which estradiol drives changes in protein expression suggests estradiol may be engaging a non-genomic mechanism of action, such as local protein synthesis. Therefore, the effect of estradiol on PSD-95 and GluN2B expression was assessed in the presence of protein synthesis inhibitor, anisomycin and gene transcription inhibitor, actinomycin D to determine whether estradiol-mediated increase of both proteins was gene transcription dependent or independent.

As in previous chapters, acute hippocampal slices prepared from male and OVX females were pre-treated with either anisomycin (40µm) or actinomycin D (20µm) 30 minutes prior to the 2 hour estradiol or DMSO exposure. The experimental set up was similar to that of the previous chapters. The slices were lysed and processed for western blotting and expression profile of PSD-95 and GluN2B was determined. Estradiol-mediated increase in PSD-95 expression was blocked in the presence of anisomycin in both male ($F_{(3,16)}=7.591, p=0.0022$, Figure 5.1A) and OVX female ($F_{(3,16)}=9.657, p=0.0007$, Figure 5.1C) hippocampus. A multiple comparisons test with Bonferroni’s correction confirmed an increase in PSD-95 expression in estradiol treated slices compared to those treated with DMSO in males ([mean ± SEM] vehicle $1.000 ± 0.08$, E2 $1.308 ± 0.08; p=0.0087$, n=5) and OVX females ([mean ± SEM] vehicle $1.000 ± 0.12$, E2 $1.495 ± 0.12; p=0.0049$, n=5). However, no expression difference was observed in slices treated with anisomycin + DMSO compared to anisomycin + estradiol in either male ([mean ± SEM] anisomycin + vehicle $1.024 ± 0.09$, anisomycin + E2
0.9705 ± 0.09; p>0.9999, n=5) nor OVX female ([mean ± SEM] anisomycin + vehicle 0.9486 ± 0.12, anisomycin + E2 0.9550 ± 0.12; p>0.9999, n=5).

Additionally, PSD-95 expression was significantly decreased in slices treated with anisomycin + DMSO (male, p=0.0166; OVX female, p=0.0020) and those treated with anisomycin + estradiol (male, p=0.0040; OVX female, p=0.0022) compared to solely estradiol treated slices in both sex.

GluN2B expression was assessed in a similar manner. Estradiol significantly increased GluN2B, which was blocked in the presence of anisomycin in both male (F(3,16)=11.10, p=0.0003, Figure 5.1B) and OVX female (F(3,16)=8.167, p=0.0016, Figure 5.1D) hippocampus. A post hoc Bonferroni’s multiple comparisons test recapitulated a significant increase in GluN2B expression between DMSO and estradiol treated slices in males ([mean ± SEM] vehicle 1.000 ± 0.05, E2 1.236 ± 0.05; p=0.0020, n=5) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.12, E2 1.357 ± 0.12; p=0.0324, n=5). Notably, GluN2B expression was not different between anisomycin + DMSO treated and anisomycin + estradiol treated slices in either males ([mean ± SEM] anisomycin + vehicle 0.9868 ± 0.05, anisomycin + E2 0.9864 ± 0.05; p>0.9999, n=5) or OVX females ([mean ± SEM] anisomycin + vehicle 1.001 ± 0.12, anisomycin + E2 0.7855 ± 0.12; p=0.5038, n=5). Estradiol treated slices exhibited higher GluN2B expression levels than those treated with anisomycin + DMSO (male, p=0.0012; OVX female p=0.0474) or anisomycin + estradiol (male, p=0.0012; OVX female p=0.0010).

As anisomycin blocked the estradiol-mediated increase of expression of synaptic proteins, the next logical step was to investigate whether these increases were maintained or blocked in the presence of actinomycin D. Increases in PSD-95 expression were corroborated in estradiol treated slices compared to DMSO treated slices, even in the presence of actinomycin D in both sexes (male, F(3,16)=20.05, p<0.0001, Figure 5.2A; OVX female, (F(3,16)=7.906, p=0.0019, Figure 5.2C). A multiple comparisons test with Bonferroni’s correction confirmed a significant increase in PSD-95 expression slices treated with estradiol when compared to DMSO treated slices in the male ([mean ± SEM] vehicle 1.000 ± 0.06, E2 1.277 ± 0.06; p=0.0026, n=5) and OVX female ([mean ± SEM] vehicle 1.000 ± 0.04, E2 1.346 ± 0.04; p=0.0026, n=5).
1.000 ± 0.08, E2 1.302 ± 0.08; p=0.0020, n=5) hippocampus. The increase in PSD-95 expression levels were maintained between actinomycin D + DMSO treated and actinomycin D + estradiol treated slices in males ([mean ± SEM] actinomycin D + vehicle 0.8427 ± 0.06, actinomycin D + E2 1.208 ± 0.06; p=0.0002, n=5) and OVX females ([mean ± SEM] actinomycin D + vehicle 0.9219 ± 0.08, actinomycin D + E2 1.193 ± 0.08; p=0.0416, n=5). Slices treated with estradiol or actinomycin D + estradiol did not reveal any difference in PSD-95 expression in either sex (p>0.9999). PSD-95 expression was found to be higher in estradiol treated slices compared to anisomycin + DMSO treated slices in both sexes (male, p<0.0001; OVX female, p=0.0031).

Similar expression profile of GluN2B was found in response to estradiol in the presence of actinomycin D. Estradiol enhanced GluN2B expression levels, and this was emulated in the presence of actinomycin D in both sexes (male, F(3,16)=10.40, p=0.0005, Figure 5.2B; OVX female, F(3,16)=9.076, p=0.0010, Figure 5.2D). Upon a multiple comparisons test with Bonferroni’s correction, estradiol substantiated the increase in GluN2B in both male ([mean ± SEM] vehicle 1.000 ± 0.10, E2 1.334 ± 0.10; p=0.0306, n=5) and OVX female ([mean ± SEM] vehicle 1.000 ± 0.11, E2 1.437 ± 0.11; p=0.0081, n=5), compared to vehicle. GluN2B expression continued to be increase in actinomycin + estradiol treated slices compared to those treated with actinomycin + DMSO in the male ([mean ± SEM] actinomycin D + vehicle 0.7647 ± 0.10, actinomycin D + E2 1.083 ± 0.10; p=0.0422, n=5) and OVX female ([mean ± SEM] actinomycin D + vehicle 1.181 ± 0.11, actinomycin D + E2 1.526 ± 0.11; p=0.0450, n=5) hippocampus. No difference in expression was observed in slices treated solely with estradiol or actinomycin + estradiol in either (male, p=0.1611; OVX female, p=0.0031). Additionally, GluN2B expression was found to be significantly higher in slices treated with estradiol compared to actinomycin D + DMSO in males (p=0.0003) but this was not the case in OVX females (p=0.2241).
Figure 5.1 Estradiol-mediated increase in PSD-95 and GluN2B expression is inhibited in the presence of anisomycin in both male and OVX female hippocampus. A-D, Representative western blots and quantification of acute hippocampal slices of male (A+B) and OVX female (C+D) hippocampal prepared from 10-12 week old mice. Slices were pre-treated with anisomycin (40 µM, 30 mins) followed by 2 hour estradiol (10 nM) or DMSO treatment and processed for western blotting. Blots were probed for either PSD-95 or GluN2B and normalised to housekeeper, β-actin. Anisomycin inhibited estradiol-mediated increases of PSD-95 in the male (A) and OVX female (C) hippocampus. Increased GluN2B expression was also inhibited in the presence of anisomycin in males (C) and OVX females (D); n=5 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, *** p = <0.001, ns = not significant.
Figure 5.2 Actinomycin D does not affect estradiol-mediated increase of PSD-95 and GluN2B expression in the male and OVX female hippocampus. A-D, Representative western blots and quantification of acute hippocampal slices of male (A+B) and OVX female (C+D) hippocampal prepared from 10-12 week old mice. Slices were pre-treated with actinomycin D (20 µM, 30 mins) followed by 2 hour estradiol (10 nM) or DMSO treatment and processed for western blotting. Blots were probed for either PSD-95 or GluN2B and normalised to housekeeper, β-actin. Estradiol mediated an increase in PSD-95 expression in the presence of actinomycin D in the male (A) and OVX female (C) hippocampus. Estradiol also continued to increase GluN2B expression in the presence of actinomycin D in both males (C) and OVX females (D); n=5 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, **** p = <0.0001, ns = not significant.
These data demonstrate that the estradiol-mediated increases in PSD-95 and GluN2B protein expression levels is dependent on a non-genomic mechanism of action. Where anisomycin blocks these increases, they continue to be facilitated by estradiol in the presence of actinomycin D. This result draws parallels from the biochemical data of SUnSET-WB (Figures 4.2 & 4.3), whereby estradiol-mediated increase in the rate of translation continued to be facilitated in the presence of actinomycin D.

**Estradiol increases PSD-95 and GluN2B expression in crude synaptosomal fractions while having no effect on mRNA levels in primary hippocampal neurons**

The lysates from male and OVX female hippocampus from the previous section encompass the whole hippocampus thus, the subcellular site which estradiol is mediating the increase in protein synthesis cannot be determined. Therefore, there was a need to investigate where in the cell estradiol increases protein synthesis. Additionally, whether estradiol also induced changes in PSD-95, GluN2B and GluA1 mRNA levels within the same time-frame were examined. Crude synaptosomal fractions were prepared from mature (DIV 27) primary rat hippocampal neurons to identify whether the estradiol-mediated increases in PSD-95 and GluN2B expression was occurring specifically at synapses. PSD-95 expression levels were found to be significantly increased ([mean ± SEM] vehicle 1.000 ± 0.22, E2 1.983 ± 0.22; t=3.052, df=4.00, p=0.0380, n=3; Figure 5.3A) in crude synaptosomal fractions from estradiol treated cells compared to vehicle. Indeed, estradiol also increased GluN2B expression levels ([mean ± SEM] vehicle 1.000 ± 0.44, E2 1.446 ± 0.44; t=7.044, df=4.00, p=0.0021, n=3; Figure 5.3B) within the same time-frame.

These data show enhanced protein levels of both PSD-95 and GluN2B in response to estradiol and so whether estradiol influences these proteins at the mRNA level was also determined. Expression of DLG4 (for PSD-95) and GRIN2B (for GluN2B) expression was assessed using a RT-qPCR for changes in response to 2 hour estradiol treatment. Estradiol induced no significant change in either DLG4 ([mean ± SEM] vehicle 2.562 ± 0.52, E2 2.353 ± 0.394 t=0.3230,
df=5.585, p=0.7584, n=4; **Figure 5.3C** or GRIN2B ([mean ± SEM] vehicle 0.7017 ± 0.15, E2 0.6896 ± 0.11; t=0.06613, df=5.548, p=0.9496, n=4; **Figure 5.3D**) expression when normalised to housekeepers ACTB, GUSB and B2M, compared to DMSO. This is in line with previous research where estradiol treatment induced no change in PSD-95 mRNA levels after 6 hours in the d-NG108-15 cell line (Akama and McEwen, 2003). Additionally, no expression change was detected in GRIA1 (for GluA1) ([mean ± SEM] vehicle 1.315 ± 0.18, E2 1.280 ± 0.15; t=0.1473, df=5.875, p=0.8878, n=4; **Figure 5.3E**) in estradiol treated neurons compared to vehicle within the same time-frame.

These results demonstrate that estradiol increases PSD-95 and GluN2B protein expression specifically at crude synaptosomes whilst having no effect on their mRNA levels. This provides further evidence that estradiol is influencing the local synthesis of PSD-95 and GluN2B. As GRIA1 expression is not sensitive to estradiol treatment, it would be a good candidate protein to investigate whether the estradiol-mediated increase in GluA1 expression in Chapter 3 could be dependent on local protein synthesis.
Figure 5.3 Estradiol increases PSD-95 and GluN2B expression in crude synaptosomal fractions but doesn’t affect the respective mRNA expression in primary hippocampal neurons. A-E, DIV 27 primary hippocampal neurons were treated with estradiol (10 nM, 2 hours) or a vehicle control. A-B, Representative western blots and quantification. Neurons were fractionated upon lysing to separate out the extra-nuclear and crude synaptosomal fractions and processed for western blotting. Blots were probed for either PSD-95 or GluN2B and normalised to housekeeper, β-actin. PSD-95 and GluN2B protein expression was increased in the crude synaptosome fractions in estradiol treated neurons; n=3 per condition. C-E, Quantification of RT-qPCR. RNA was extracted from the neurons and measured for difference in DLG4, GRIN2B and GRIA1 expression. No differences were found in the relative abundance of DLG4, GRIN2B and GRIA1 transcripts between estradiol or vehicle treated neurons within the same time-frame. Expression level was normalised to the geometric mean of three reference genes (ACTB, GUSB and B2M); n=4 per condition. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05, **p = <0.01, ns = not significant.
PSD-95 and GluN2B are increased specifically within dendritic spines and along dendrites following estradiol treatment

Biochemical detection of increased PSD-95 and GluN2B expression within crude synaptosome fractions suggests that estradiol is increasing the presence of these proteins specifically at synaptic terminals. Consequently, Sarkar et al.'s (2010) work showing increased mRNA translation along dendrites in response to estradiol over 1 hour, suggests that estradiol has the potential to rapidly initiate translation of dendritic mRNA. Taken together with the evidence of the presence of a large number of mRNAs and several translation initiation proteins at dendrites (Steward and Schuman, 2001), the estradiol-mediated increase of both PSD-95 and GluN2B could be targeted to dendrites. To investigate the subcellular location of protein increases, primary hippocampal neurons were transfected as previously at DIV 12 with eGFP and cultured until DIV 20-21. Neurons were treated with DMSO or estradiol for 2 hours and subsequently fixed and immunostained for PSD-95 and GluN2B. Neurons were then imaged on a confocal microscope and protein intensity was measured within spines and along dendrites across approximately 100 µm sections of secondary and tertiary primary dendrites, as detailed in Chapter 2. PSD-95 expression was increased within spines ([mean intensity ± SEM] vehicle 0.9399* ± 0.05, E2 1.207 ± 0.11; t=2.264, df=17.28, p=0.0367, n=13 per condition; Figures 5.4A+B; *one cell data removed following Grubbs’s test for outliers) and dendrites ([mean intensity ± SEM] vehicle 1.000 ± 0.08, E2 1.554 ± 0.17; t=2.898, df=15.48, p=0.0108, n=14, 12 respectively; Figures 5.4A+C) following estradiol treatment. Figure 5.4A demonstrates increases in PSD-95 intensity specifically at spines (white arrows) and along dendrites (yellow arrows). Specifically, PSD-95 can be visualised within spine heads of estradiol treated neurons represented by colocalization (in white) of PSD-95 (magenta) in GFP positive spine (green) (Figure 5.5D). Estradiol also increased GluN2B expression at spines ([mean intensity ± SEM] vehicle 0.9742 ± 0.04, E2 1.509 ± 0.23; t=2.331, df=10.75, p=0.0403; n=13, 11 respectively; Figures 5.5A+B). However, no GluN2B expression changes were detected along the dendrites in estradiol treated neurons (p=0.5438; vehicle 1.000 ± 0.13, E2 1.133 ± 0.17; t=0.6171, df=21.00, p=0.5438; n=14, 12 respectively, Figures 5.5A+C) compared to vehicle. Figures 5.4A+D
demonstrate increased PSD-95 intensity within spines (white arrows) and dendrites (yellow arrows). Figure 5.5A+D demonstrate increased GluN2B intensity within spines (white arrows) whereas, there is no pronounced increase at dendrites (yellow arrows).

Figure 5.4 PSD-95 is increased along dendrite and within dendritic spines in primary hippocampal neurons. A+D, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) or DMSO and immunostained for GFP (green) and PSD-95 (magenta). B-C, Quantification of A. Estradiol increased PSD-95 intensity within dendritic spines (B) and along dendrites (C). D, Orthogonal projections confirmed localisation of PSD-95 within spines in estradiol treated neurons; n= 13-14.
Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * \( p = <0.05 \). Arrowheads denote PSD-95 localised at dendrites (yellow) and dendritic spines (white). Co-localisation of PSD-95 within GFP positive regions is shown in white. Scale bars = 10 µm (A), 5 µm (D).

Figure 5.5 *GluN2B is increased within dendritic spines but not extrasynaptically in primary hippocampal neurons*. A+D, Representative confocal images (60x) of GFP transfected DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) and immunostained for GFP (green) and GluN2B (magenta). B-C, Quantification of A. Estradiol increased GluN2B intensity within dendritic spines (B) but not along dendrites (C). D, Orthogonal projections confirmed localisation of GluN2B within spines in estradiol treated neurons; n= 13-14. Unpaired
student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p < 0.05. Arrowheads denote PSD-95 localised at dendrites (yellow) and dendritic spines (white). Co-localisation of GluN2B within GFP positive regions is shown in white. Scale bars = 10 µm (A), 5 µm (D).

These data confirm that estradiol is increasing PSD-95 and GluN2B expression in close proximity to dendritic spines within 2 hours. As estradiol has no significant effect on their respective mRNA levels, these results suggest that estradiol may be engaging dendritic mRNA to translate these proteins locally.

mTOR is required for estradiol-dependent increase of PSD-95 in both male and OVX females

Chapter 4 demonstrated that mTOR was needed for estradiol-dependent protein synthesis in the male but not OVX female hippocampus. However, SUnSET does not give out individual protein readout and so although mTOR may be important for global protein synthesis in males, whether this signalling pathway is needed for increasing PSD-95 expression levels was specifically determined. Pre-treated acute hippocampal slices with rapamycin (1µm, 30 minutes) followed by 2 hour estradiol or DMSO exposure from males and OVX females were lysed and processed for western blotting and expression profile of PSD-95. Interestingly, rapamycin blocked the estradiol-mediated increase of PSD-95 expression in both male ($F_{(3,20)}=9.955$, p=0.0003, Figure 5.6A) and OVX female ($F_{(3,20)}=8.206$, p=0.0009, Figures 5.6B) hippocampus. A post hoc Bonferroni’s multiple comparison test confirmed the increase of PSD-95 expression in estradiol treated slices compared to vehicle in both males ([mean ± SEM] vehicle 1.000 ± 0.07, E2 1.356 ± 0.07; p=0.0002, n=6) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.07, E2 1.356 ± 0.07; p=0.0006, n=6). This increase was attenuated in the presence of rapamycin in estradiol treated slices in both males ([mean ± SEM] rapamycin + vehicle 1.218 ± 0.67, rapamycin + E2 1.147 ± 0.67; p>0.9999, n=6) and OVX females ([mean ± SEM] vehicle 1.153 ± 0.07, E2 1.115 ± 0.07; p>0.9999, n=6). PSD-95 expression was significantly higher in slices treated with estradiol compared to rapamycin + estradiol treated hippocampal slices in both
sexes (males \( p=0.0267 \); OVX female, \( p=0.0218 \)). Slices treated with rapamycin + DMSO also increased PSD-95 expression compared to DMSO alone in males (\( p=0.0231 \)), which was not observed between OVX female slices (\( p=0.2993 \)).

Thus, the current data illustrates that mTOR is specifically important in increasing PSD-95 expression in males but also OVX females. This suggests that although mTOR is not required to increase global protein synthesis in the OVX female hippocampus, it is required to increase PSD-95 expression and possibly other proteins.

**Figure 5.6** mTOR is required for estradiol-mediated increase in PSD-95 expression in the male and OVX female hippocampus but mTOR is required for estradiol-mediated increase in GluN2B only in the male but not OVX female hippocampus. A-D, Representative western blots and quantification of acute
hippocampal slices of male (A+C) and OVX female (B+D) hippocampal prepared from 10-12 week old mice. Slices were pre-treated with rapamycin (1 \( \mu M \), 30 mins) followed by 2 hour estradiol (10 nM) or DMSO treatment and processed for western blotting. Blots were probed for either PSD-95 or GluN2B and normalised to housekeeper, \( \beta \)-actin. Rapamycin inhibited estradiol-mediated increases of PSD-95 in the male (A) and OVX female (B) hippocampus. Increased GluN2B expression was also inhibited in the presence of rapamycin in males (C) but not OVX females (D); n=5 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; * \( p = < 0.05 \), ** \( p = < 0.01 \), *** \( p = < 0.001 \), **** \( p = < 0.0001 \), ns = not significant.

**Estradiol increases GluN2B expression independent of mTOR in OVX females but not males**

It was next identified whether mTOR was critical for increasing expression levels of GluN2B in both of the male and OVX female hippocampus. The same hippocampal slices treated with DMSO and estradiol in the presence or absence of rapamycin were processed for western blotting to assess the expression profile of GluN2B. Interestingly, mTOR blocked the increase in GluN2B expression in the male (\( F_{(3,16)}=6.932, p=0.0033 \), **Figure 5.6C**) but not the OVX female (\( F_{(3,16)}=9.305, p=0.0009 \), **Figure 5.6D**) hippocampus. A multiple corrections test with Bonferroni’s correction multiple comparisons test recapitulated increased GluN2B expression in estradiol treated slices compared to vehicle in both males ([mean ± SEM] vehicle 1.000 ± 0.24, E2 2.049 ± 0.24; \( p=0.0025 \), n=5) and OVX females ([mean ± SEM] vehicle 1.000 ± 0.13, E2 1.554 ± 0.13; \( p=0.0030 \), n=5). This increase in GluN2B expression was attenuated in rapamycin + estradiol treated slices compared to rapamycin + DMSO in the male ([mean ± SEM] rapamycin + vehicle 1.405 ± 0.24, rapamycin + E2 1.322 ± 0.24; \( p>0.9999 \), n=5) but not OVX female ([mean ± SEM] rapamycin + vehicle 1.004 ± 0.13, rapamycin + E2 1.392 ± 0.13; \( p=0.0390 \), n=5) hippocampus. This suggested that mTOR was not required for estradiol to increase GluN2B expression in the OVX females. In fact, GluN2B expression was found to be increased in slices treated with rapamycin + estradiol compared to vehicle (\( p=0.0370 \)). When compared to rapamycin + estradiol treated slices, GluN2B expression was significantly higher.
in slices treated solely treated with E2 in males (p=0.0433) but no significant change was found in OVX females (p=0.6040). Additionally, GluN2B expression was found to be profoundly increased in slices treated with estradiol compared to those treated with rapamycin + DMSO in the OVX female (p= 0.0031) hippocampus.

These data show that estradiol increased GluN2B expression in a sexually dimorphic manner. mTOR signalling was not required for estradiol to increase GluN2B expression in the OVX female hippocampus, unlike PSD-95. Critically, mTOR is required to increase GluN2B expression in the male hippocampus. These results mirror the findings from Chapter 4, whereby the increase in estradiol-mediated global protein synthesis is mTOR dependent in males but not OVX females. Interestingly, mTOR is important in regulating increases in PSD-95 in both sexes suggesting that the mTOR signalling pathway is critical in mediating estradiol-dependent increases in select proteins in OVX females proposing the contribution of a second pathway in the OVX female hippocampus.

RPS6 is phosphorylated in males but not OVX females

A consequence of the activation of mTOR is the subsequent phosphorylation of RPS6 (p-RPS6) through S6K1/2 (Meyuhas, 2015). Estradiol increases p-RPS6 within 10 minutes in primary hippocampal neurons, which remains phosphorylated after 1 hour (Sarkar et al., 2010). p-RPS6 has been associated with LTP in the CA1 (Cammalleri et al., 2003; Panja et al., 2009; Tsokas et al., 2005) in addition to having a regulatory role in translation initiation and protein synthesis (Thomas et al., 1982). Phosphorylation at serine sites 235 and 236 (Ser235/236) have been demonstrated to be shown to be MAPK/ERK dependent near active synapses whereas, phosphorylation at serine sites 240 and 244 (Ser240/244) are predominantly mTOR dependent in dendrites (Pirbhoy et al., 2017). This suggests a convergence at RPS6 for both signalling pathways. To determine the contribution of p-RPS6 within the current study, the activation of both Ser235/236 and Ser240/244 was assessed in the male and OVX female hippocampus.
Employing the hippocampal slice lysates treated with DMSO or estradiol with or without pre-treatment of rapamycin from the previous 2 results sections, p-RPS6 was measured through western blotting. Estradiol increased phosphorylation of RPS6 Ser\(^{235/236}\), which subsequently blocked by rapamycin (\(F_{(3,16)}=40.99, p<0.0001\), Figure 5.7A) and ser\(^{240/244}\) (\(F_{(3,16)}=90.76, p<0.0001\), Figure 5.7B) in the male hippocampus. However, no change in RPS6 phosphorylation was observed at either phosphorylation sites in the OVX female hippocampus (Ser\(^{235/236}\): \(F_{(3,20)}=12.5, p<0.0001\), Figure 5.7C, Ser\(^{240/244}\): \(F_{(3,20)}=109.1, p<0.0001\), Figure 5.7D); rapamycin categorically attenuated the phosphorylation.

A Bonferroni corrected multiple comparisons test illustrated a significant increase in p-RPS6 at Ser\(^{235/236}\) ([mean ± SEM] vehicle 1.000 ± 0.10, \(\text{E2} 1.360 ± 0.10\); \(p=0.0203, n=5\)) and Ser\(^{240/244}\) ([mean ± SEM] vehicle 1.000 ± 0.08, \(\text{E2} 1.308 ± 0.08\); \(p=0.0051, n=5\)) in estradiol treated slices compared to vehicle in males. Whereas, no change in RPS6 activation was detected in either Ser\(^{235/236}\) ([mean ± SEM] vehicle 1.000 ± 0.13, \(\text{E2} 0.8405 ± 0.13\); \(p>0.9999, n=5\)) or Ser\(^{240/244}\) ([mean ± SEM] vehicle 1.000 ± 0.06, \(\text{E2} 0.8977 ± 0.06\); \(p=0.5859, n=5\)) in OVX female slices treated with estradiol. Expectedly, p-RPS6 was significantly decreased in rapamycin + DMSO and rapamycin + estradiol treated slices compared to vehicle at Ser\(^{235/236}\) ([mean ± SEM] rapamycin + vehicle 0.3291 ± 0.10, \(p<0.0001, n=5\); rapamycin + \(\text{E2} 0.4826 ± 0.10\), \(p=0.0009, n=5\)) and Ser\(^{240/244}\) ([mean ± SEM] rapamycin + vehicle 0.2983 ± 0.08, \(p<0.0001, n=5\); rapamycin + \(\text{E2} 0.3085 ± 0.08\), \(p<0.0001, n=5\)). This was parallel in the OVX female hippocampus, RPS6 activation was found to be attenuated in rapamycin + DMSO and rapamycin + estradiol treated slices compared to vehicle at both Ser\(^{235/236}\) ([mean ± SEM] rapamycin + vehicle 0.3200 ± 0.13, \(p=0.0003, n=5\); rapamycin + \(\text{E2} 0.4036 ± 0.13\), \(p=0.0013, n=5\)) and Ser\(^{240/244}\) ([mean ± SEM] rapamycin + vehicle 0.2026 ± 0.06, \(p<0.0001, n=5\); rapamycin + \(\text{E2} 0.1964 ± 0.06\), \(p<0.0001, n=5\)). Indeed, p-RPS6 at Ser\(^{235/236}\) was also significantly lower in rapamycin + DMSO (male, \(p<0.0001\); OVX female, \(p=0.048\)) and rapamycin + estradiol treated (male, \(p<0.0001\); OVX female, \(p=0.0209\)) slices when compared to slices treated with estradiol. This was also the case for p-RPS6 at Ser\(^{240/244}\) compared to estradiol treated slices (male, rapamycin + DMSO & rapamycin + E2, \(p<0.0001\) for both; OVX female, rapamycin + DMSO & rapamycin + E2, \(p<0.0001\) for both).
Figure 5.7 *Estradiol increases phosphorylation of ribosomal protein S6 (RPS6) in the male, but not OVX female hippocampus.* A-D, Representative western blots and quantification of acute hippocampal slices of male (A+B) and OVX female (C+D) hippocampus prepared from 10-12 week old mice. Slices were pre-treated with rapamycin (1 µM, 30 mins) followed by 2 hour estradiol (10 nM) or DMSO treatment and processed for western blotting. Blots were probed for either RPS6 phosphorylation at serine sites 235 (Ser^{235/236}) and 236 or serine sites 240 and 244 (Ser^{240/244}) and normalised to total RPS6 expression. Estradiol increased phosphorylation of both Ser^{235/236} and Ser^{240/244} in the male, but not the OVX female, hippocampus. Rapamycin blocked the phosphorylation of all the serine sites in both males and OVX females; n=5 per condition. One-way ANOVA,
Collectively, these data show that estradiol phosphorylated RPS6 at Ser^{235/236} and Ser^{240/244} in males within 2 hours, which complement the findings that mTOR is required for estradiol-mediated increases in global protein synthesis in the male hippocampus. Critically, estradiol does not activate either Ser^{235/236} or Ser^{240/244} in the OVX female hippocampus within the same time-frame. Amalgamating this finding with the findings from Chapter 4, estrogen engages a different pathway to increase GluN2B levels in the OVX but requires mTOR to increase PSD-95. Thus, estradiol may need increased phosphorylation of RPS6 to mediate the increase of specific proteins in the OVX hippocampus. Yet, this sensitivity may not be picked up through western blotting.

Estradiol rapidly increases the rate of translation along dendrites and juxtaposing synaptic regions

Data from Chapter 4 showing that increased AHA-tagged newly synthesised proteins localised to spines suggests that nascent proteins may be synthesised nearby. In concert, estradiol increased PSD-95 and GluN2B expression near and within spines. Thereby, a subset of these proteins could be newly synthesised. To investigate this, SUNSET-ICC was employed to determine the subcellular location of active translation sites in estradiol treated neurons. As puromycin incorporates onto elongating polypeptide chains, the mechanism can provide a notion of where in the neuron puromycin terminates the elongation. Thereby, demonstrating where increased translation occurs in neurons following estradiol treatment. Primary hippocampal neurons (DIV20-21) were treated for 2 hours with either DMSO or estradiol over 2 hours as previously, subsequently immunostained with the neuronal marker MAP2 and puromycin. Neurons were imaged using structured illumination microscopy (SIM) to provide a better resolution than traditional confocal microscopy. Puromycin puncta number and area was analysed along dendrites approximately 50 µm in length. Puromycin
puncta number and area was also measured in the spine area, which was defined by measuring 2 µm either side of the dendrite, as detailed in Chapter 2.

Puromycin puncta number was increased in estradiol treated neurons in crude synaptic regions (per 10 µm [mean ± SEM] vehicle 27.73 ± 2.45, E2 43.96 ± 2.30; t=4.830, df=26.00, p<0.0001, n=15, 13 respectively; Figures 5.8A+B) demonstrating increased rate of translation compared to a vehicle control. Upon closer analysis, there is increased puromycin puncta specifically along dendrites (per 10 µm [mean ± SEM] vehicle 8.031 ± 0.97, E2 12.51 ± 0.83; t=3.505, df=25.80, p=0.0017, n=15, 13 respectively; Figures 5.8A+C) and in spine regions (per 10 µm [mean ± SEM] vehicle 19.70 ± 1.67, E2 31.46 ± 2.02; t=4.481, df=24.30, p=0.0002, n=15, 13 respectively; Figures 5.8A+D). Estradiol also increased puncta area in the synaptic area ([mean ± SEM] vehicle 0.04146 ± 0.001, E2 0.4719 ± 0.001; t=3.024, df=25.98, p=0.0056, n=15, 13 respectively; Figures 5.8A+E). This is specifically pronounced along dendrites ([mean ± SEM] vehicle 0.04027 ± 0.002, E2 0.4628 ± 0.002; t=2.579, df=26.00, p=0.0159, n=15, 13 respectively; Figures 5.8A+F) and synaptic regions ([mean ± SEM] vehicle 0.04183 ± 0.002, E2 0.4629* ± 0.001; t=2.304, df=24.55, p=0.0299, n=15, 12 respectively; Figures 5.8A+G; *one cell data removed following Grubb’s test for outliers). Figure 5.8A demonstrates increased puromycin puncta number and area within spine regions (white arrows) and dendrites (yellow arrows).

Collectively, these data firstly provide further evidence that puromycin incorporation is promoted in estradiol treated neurons. Thus, there is an increase in the rate of translation specifically along dendrites and within spine areas, confirming data from SUNSET-WB in crude synaptosomes in Chapter 4 (Figure 4.6). Increased puncta size could suggest that multiple proteins undergoing elongation could be in close proximity, that may not be detected through SIM in neurons treated with estradiol.
Figure 5.8 Estradiol increases puromycin-tagged elongating proteins, representing active translation sites, along dendrites and within dendritic spines in primary hippocampal neurons. A, Representative SIM images (100x) of DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) or DMSO, incubated with puromycin (10 µg/mL, last 10 minutes). Neurons were immunostained for neuronal marker MAP2 (magenta) and puromycin (green; SUnSET-ICC). B-G, Quantification of A. Estradiol increases puromycin puncta number (B-D) and puncta size (E-G) within synaptic areas (B-E). Specifically, increased puromycin puncta number and size was found in dendrites (C+F) and within spine regions (D+G); n = 13-15. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05, ** p = <0.01, *** p = <0.001, **** p = < 0.0001, ns = not significant. Arrowheads denote puromycin localised at dendrites (yellow) and within spine regions (white). Co-localisation of puromycin within MAP2 positive regions is shown in white. Scale bar = 5 µm.
RPS10 is increased along dendrites and within spine regions

As estradiol increases the rate of translation along dendrites and the juxtaposing spine regions, it was reasoned that estradiol may affect translational machinery. Polyribosomes have previously been shown to fluctuate during the female rat estrous cycle (McCarthy and Milner, 2003). Expressions of both ribosomal S3 protein, member of the smaller 40S ribosomal subunit, and ribosomal p-proteins, part of the larger 60S ribosomal subunit, were increased during estrus, when estrogen levels are higher, compared to diestrus and proestrus in the hippocampal CA1 stratum radiatum. Since, no further interaction between estradiol and ribosomal proteins have been documented. Thus, given the nature of this study, ribosomal protein S10, a component of the 40S ribosomal subunit, was assessed in response to 2 hour estradiol treatment.

Employing the same experimental set up as SUnSET-ICC, treated primary hippocampal neurons were additionally co-immunostained with RPS10. All images were acquired with SIM as above, coupled with the SUnSET-ICC experiment. RPS10 puncta number and size were determined using the same regions of interest (ROI) as puromycin. This also allowed the investigation of any co-localisation between puromycin and RPS10, which will be detailed in the next results section. Estradiol treatment significantly increased RPS10 puncta number in the crude synaptic region (per 10 µm [mean ± SEM] vehicle 19.57 ± 1.75, E2 28.26 ± 3.02; t=2.487, df=19.54, p=0.0221, n=15, 13 respectively; Figures 5.9A+B) compared to DMSO. When assessed separately, increased RPS10 puncta could be visualised along dendrites (per 10 µm [mean ± SEM] vehicle 4.766 ± 0.38, E2 6.886 ± 0.78; t=2.653, df=18.63, p=0.0159, n=15, 13 respectively; Figures 5.9A+C) spine regions (per 10 µm [mean ± SEM] vehicle 14.41 ± 1.33, E2 21.38 ± 2.50; t=2.463, df=18.50, p=0.0238, n=15, 13 respectively; Figures 5.9A+D) compared to vehicle suggesting estradiol was increasing RPS10 expression locally at dendrites. Whether this is solely the RPS10 positive 40S ribosomal subunits or whether it represents increases of functional ribosomes cannot be determined.

The increase in RPS10 puncta number was in concert with increases in puncta size. Estradiol treated neurons expressed larger RPS10 puncta area compared
to vehicle ([mean ± SEM] vehicle 0.04205 ± 0.002, E2 0.05165 ± 0.003; t=2.594, df=20.89, p=0.0170, n=15, 13 respectively; Figures 5.9A+E). This signature was observed specifically along dendrites ([mean ± SEM] vehicle 0.04031 ± 0.002, E2 0.05059 ± 0.004; t=2.544, df=16.77, p=0.0211, n=15, 13 respectively; Figures 5.9A+F) and within spine regions ([mean ± SEM] vehicle 0.04258 ± 0.002, E2 0.05162 ± 0.003; t=2.391, df=21.99, p=0.0258, n=15, 13 respectively; Figures 5.9A+G). Therefore, estradiol not only increased the distribution of 40S ribosomal subunits, but this was coupled with an increase in RPS10 puncta area. Figure 5.9A demonstrates increased RPS10 puncta number and area within spine regions (white arrows) and dendrites (yellow arrows).

Taken together, these data suggest that RPS10 distribution is increased along dendrites and spine regions in estradiol treated neurons. It is unknown whether these are active ribosomes nevertheless, it illustrates that estradiol is interacting with ribosomal machinery with this time-frame. RPS10 puncta area was also found increased in the same time-frame in estradiol treated neurons. It can be reasoned that this could represent clustering of RPS10 positive ribosomal subunits that may be actively translating mRNA. Nevertheless, this echoes the findings of increased dendritic polyribosomal accumulation during estrus (McCarthy and Milner, 2003).
Figure 5.9 Ribosomal protein S10 (RPS10) is increased along dendrites and within spine regions following 2 hour estradiol treatment in primary hippocampal neurons. A, Representative SIM images (100x) of DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) or DMSO and immunostained for neuronal marker MAP2 (magenta) and RPS10 (green). B-G, Quantification of A. Estradiol increased RPS10 puncta number (B-D) and puncta size (E-G) within synaptic areas (B-E). Specifically, increased RPS10 puncta number and size was found in dendrites (C+F) and within spine regions (D+G); n= 13-15. Unpaired student's t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05. Arrowheads denote RPS10 localised at dendrites (yellow) and within spine regions (white). Co-localisation of RPS10 within MAP2 positive regions is shown in white. Scale bar = 5 µm.
Co-localisation of puromycin-tagged nascent proteins and RPS10 is increased

Increased puromycin and RPS10 puncta were observed in the same chosen ROIs along estradiol treated neurons. Thus, it was determined whether puromycin and RPS10 would colocalise with each other. It can be reasoned that co-localisation between puromycin and RPS10 may be indicative of an actively translating ribosome where puromycin has terminated the elongation of polypeptide chain in RPS10 positive ribosomes. To investigate this, RPS10 puncta were assessed for co-localisation with puromycin by projecting identified RPS10 puncta ROIs into the respective puromycin channel from the same neuron. This would allow to measure how much RPS10 would localise with puromycin. Estradiol drove an increase in RPS10 co-localisation with puromycin in synaptic regions (per 10 µm [mean ± SEM] vehicle 17.56 ± 1.67, E2 25.60 ± 3.13; t=2.265, df=18.50, p=0.0358, n=15, 13 respectively; Figures 5.10A+B) compared to vehicle. This was observed both along dendrites (per 10 µm [mean ± SEM] vehicle 4.649 ± 0.38, E2 6.747 ± 0.73; t=2.537, df=18.26, p=0.0205, n=15, 13 respectively; Figures 5.10A+C) and spine regions (per 10 µm [mean ± SEM] vehicle 12.54 ± 1.28, E2 18.85 ± 2.54; t=2.265, df=18.50, p=0.0393, n=15, 13 respectively; Figures 5.10A+D) in estradiol treated neurons. Figure 5.10A depicts high levels of co-localisation of RPS10 and puromycin in estradiol treated neurons compared to DMSO treated neurons, specifically at synaptic regions (white arrows) and along dendrites (yellow arrows).

These data illustrate that estradiol treated neurons encompass increased co-localisation of RPS10 with puromycin along dendrites and around spine regions. Given that puromycin tags elongating polypeptide chains, co-localisation with RPS10 could suggest an active translation site. Increased co-localisation could be visualised in both dendrites and spine regions. This corroborates previous data showing AHA-tagged newly synthesise proteins localised in the same subcellular compartments as RPS10/puromycin co-localisation. Thus, a scenario could be that estradiol may be influencing protein translation, by directly or indirectly interacting with ribosomal machinery, and inducing dendritic protein synthesis.
Figure 5.10 Estradiol increases co-localisation of RPS10 with puromycin along dendrites and within spine regions following 2 hour estradiol treatment in primary hippocampal neurons. A, Representative SIM images (100x) of DIV20 primary hippocampal neurons treated with estradiol (10 nM, 2 hours) or DMSO and immunostained for neuronal marker RPS10 (green) and puromycin (magenta). B-D, Quantification of A. Estradiol increased the co-localisation between RPS10 and puromycin puncta within synaptic areas (B). This was specifically pronounced along dendrites (C) and within spine regions (D); n= 13-15. Unpaired student’s t-tests, Welch’s correction; Error bars represent mean ± SEM; * p = <0.05. Arrowheads denote RPS10 localised at dendrites (yellow) and within spine
regions (white). Co-localisation of RPS10 puncta with puromycin puncta is shown in white. Scale bar = 5 µm.

DISCUSSION

Summary of results

This chapter tested whether estradiol-induced increase expression of PSD-95 and GluN2B was occurring via a local protein synthesis mechanism. Both proteins were found to be increased independently of gene transcription. This is consistent with the data in Chapter 4 that shows estradiol modulates a local protein synthesis mechanism. Thus, the increase in PSD-95 and GluN2B, and other proteins that have not been assessed, are the likely result of this. Estradiol dependent increase in PSD-95 was dependent on mTOR in both sexes however, mTOR was critical in increasing GluN2B only in males but not OVX females. These data further complement the notion that estradiol utilises distinct signalling mechanisms in both males and OVX females within this time-frame. Additionally, PSD-95 and GluN2B expression was found to specifically be increased dendritic spines and along dendrites. Within the same time-frame, estradiol increases the localisation of RPS10 and the active sites of translation along dendrites. This suggests that estradiol may be engaging protein translation machinery available locally to increase PSD-95 and GluN2B expression.

Estradiol increases expression of PSD-95 and GluN2B in a local protein synthesis dependent manner at dendritic spines

Estradiol increased the protein expression of both PSD-95 and GluN2B within dendritic spines and PSD-95 along dendrites. However, the fraction of these proteins detected that represent newly synthesised proteins cannot be confirmed with the existing experimental setup. Nevertheless, multiple lines of evidence from the current and previous chapter support the scenario that estradiol may be mediating the synthesis of PSD-95 and GluN2B at or near synapses. Imaging data illustrated that newly synthesised proteins are present along dendrites and within dendritic spines in estradiol treated neurons (Figures 4.7 & 4.12).
Biochemically, both PSD-95 and GluN2B were found to be enriched in the crude synaptosomal fraction following estradiol treatment, and it had no effect on their mRNA levels. As PSD-95 and GluN2B mRNAs are dendritically localised (Cajigas et al., 2012; Miyashiro et al., 1994), it is plausible that estradiol could employ these pools of existing PSD-95 and GluN2B mRNA transcripts to synthesize these proteins at, or near, spines. In support, estradiol has previously been shown to induce dendritic translation, as assessed by examining GFP expression based on a CAMKIIα 3′-UTR reporter, within a similar time-frame (Sarkar et al., 2010). SUnSET-ICC confirmed that estradiol increases active translation sites, and that the site translation is occurring is near spines and within dendrites. Therefore, estradiol has the capacity to stimulate the initiation of dendritic mRNA translation if the mRNA is already present. Critically, increases in both PSD-95 and GluN2B expression was blocked by a translation inhibitor yet, continued to be facilitated by estradiol in the presence of a gene transcription inhibitor. In line with this, SUnSET-ICC, SUnSET-WB (Figures 4.1 & 4.6) and FUNCAT (Figure 4.7) all confirm the temporal profile within which estradiol mediates these events. Thus, collectively these data indicate that estradiol increased expression of PSD-95 and GluN2B along dendrites and at or near spines, which resulted in an increase of both at synapses. PSD-95 has an important role in synapse formation (Washbourne et al., 2002), spine stabilisation (Chubykin et al., 2007) and AMPAR recruitment to synapses (Ehrlich and Malinow, 2004; Schnell et al., 2002) and GluN2B-containing synaptic NMDARs are implicated in LTP (Barria and Malinow, 2005; Berberich et al., 2007; Tang et al., 1999). Consequently, estradiol’s ability to increase two proteins implicated in synaptic plasticity provides a or several mechanisms by which estradiol may be regulating hippocampal synaptic plasticity.

Given the importance of PSD-95 in synaptic plasticity, the effect of estradiol in mediating the increase of new PSD-95 has been investigated before. As mentioned previously, new PSD-95 protein levels were detected following 6 hour estradiol treatment (10 nM) in a neuroblastoma cell line (Akama and McEwen, 2003). This was shown to be independent of gene transcription as actinomycin D blocked the increase in new PSD-95 complimented by no changes in mRNA levels. These data support the current results as they directly measured new PSD-95 protein expression levels. Although, this was carried out in a
neuroblastoma cell line through immunoprecipitation, the study lacked the specificity of investigating this within the hippocampus, where the majority of estradiol research has shown regulation of structural plasticity. Moreover, because of the methods used, the study did not specify where the newly synthesised PSD-95 was targeted. However, the time-frame of that observation supports the current study.

The observation of estradiol increasing active translation sites along dendrites may indicate that estradiol could be engaging machinery readily available at dendrites through secondary pathways. Previously, fluctuations of estrogen levels during the rat estrous cycle has been shown to trigger polyribosome accumulation at dendrites (McCarthy and Milner, 2003). However, estradiol's interaction with ribosome expression at dendrites has not since been investigated. Within the current study, increased RPS10 distribution is observed along dendrites and near spines supporting the findings from McCarthy & Milner (2003). Additionally, estradiol also increases the size of RPS10 puncta. A possible scenario is that estradiol could be promoting the trafficking of multiple RPS10 positive ribosomes to the dendrite to initiate the translation of target mRNA transcripts. Alternatively, another scenario is that estradiol may be activating stalled ribosomes. In neurons, RNA granules, comprised of non-translating ribosomes containing translationally repressed synaptic mRNA, can be transported to synapses and dwell there until an external stimuli activates these ribosomes to complete the initiation of translation (Batish et al., 2012; Costa-Mattioli et al., 2009; Krichevsky and Kosik, 2001). Recently, Graber et al. (2017) elegantly showed that some of these mRNAs are in fact translationally stalled at elongation or termination stage of translation. Therefore, this scenario provides a more rapid time-scale at which target proteins could be made. This would be ideal in a situation where there is increased depletion of a target protein. This would allow estradiol to bypass the translation initiation of pre-existing mRNA transcripts by activating a ribosome stalled at either elongation or termination of a target protein. Although RPS10 distribution is increased along dendrites, whether the RPS10 is part of ribosomal complex cannot be determined. Therefore, it is not known whether these contain mRNA or not as PSD-95 or GluN2B mRNA localisation was not observed. Alternatively, the location of estradiol-mediated translation was assessed through SUnSET-ICC.
This shows that it is occurring both at dendrites and near spines. Consistent with this, RPS10 is increased within the same subcellular compartments, and there is increased co-localisation of puromycin with RPS10. Some puromycin puncta were also observed to not co-localise with RPS10, a possible explanation for this could be that the puromycin-labelled proteins may have undergone premature termination and thus, deviated away from any ribosomal protein complexes. Although, how fast this can occur is unknown. Alternatively, puromycin-labelled proteins may be part of a ribosomal complex which, does not include RPS10. Taken together, more studies are warranted to fully elucidate the interaction between estradiol and ribosomal distribution for example, using approaches such as Translating Ribosome Affinity Purification (TRAP) to label polyribosomes containing translating mRNAs (Heiman et al., 2014).

mTOR is required for estradiol to increase the expression of specific proteins

A reoccurring theme amidst the data presented in this thesis is that estradiol behaves in a sexually dimorphic manner in the rodent hippocampus. mTOR was shown to be required to induce estradiol-dependent global protein synthesis in the male but not female hippocampus (Figure 4.4). Furthermore, mTOR was also required for estradiol-induced increase in PSD-95 and GluN2B expression levels in the male hippocampus and interestingly, only PSD-95 in OVX female hippocampus. As SUnSET is a measure of global protein synthesis, it is likely that multiple signalling pathways are involved in mediating estradiol-mediated protein synthesis. Therefore, it is possible that mTOR is required for mediating a proportion of estradiol-mediated protein synthesis, but this cannot be detected using SUnSET-WB. Thus, looking at specific proteins provides a greater specificity to decipher whether mTOR is needed for estradiol to mediate the translation of that specific protein. Nevertheless, the data also showed that mTOR was not required for estradiol to increase GluN2B in the OVX female hippocampus. This further confirms that another signalling pathway could be involved in mediating estradiol-mediated global protein synthesis, and the translation of GluN2B in the OVX hippocampus within the current time-frame.
Estradiol phosphorylated RPS6, a downstream target of mTOR, at both Ser$^{235/236}$ and Ser$^{240/244}$ in the male hippocampus, but not the OVX female hippocampus. This suggests that the phosphorylation of RPS6 may contribute to the mTOR-dependent increase in PSD-95 and GluN2B in the male hippocampus. This supports the data showing mTOR was required for increasing estradiol-mediated global protein synthesis in males. A potential route estradiol could employ is activating RPS6 through mTOR. In support, RPS6 activation at Ser$^{235/236}$ has recently been shown to be induced along dendrites and particularly near active synapses in the hippocampus, suggesting that it may have a critical role in the translation of mRNA present near synapses (Pirbhoy et al., 2016). Furthermore, RPS6 has been reported to be trafficked into dendritic spines following LTP induction in the dentate gyrus (Nihonmatsu et al., 2015), further proposing a potential role for RPS6 in dendritic protein synthesis. Interestingly, Ser$^{235/236}$ phosphorylation has been shown to be MAPK/ERK dependent near active synapses whereas, phosphorylation at Ser$^{240/244}$ is predominantly mTOR dependent in dendrites (Pirbhoy et al., 2017). However, estradiol did not increase phosphorylation of either Ser$^{235/236}$ or Ser$^{240/244}$ in the OVX female hippocampus. Therefore, although mTOR is needed to increase PSD-95 in the OVX females, it would not utilise the phosphorylation of RPS6 within the time-frame. mTOR also phosphorylates 4EBP1, which prevents eIF4E from initiating translation by suppressing its association with the mRNA ‘cap’ (Hoeffer and Klann, 2010). Phosphorylation through mTOR releases eIF4E to resume this binding and initiation of translation. Thus, estradiol could possibly be increasing PSD-95 through mTOR via 4EBP1. ERK is also able to phosphorylate eIF4E (Banko et al., 2004; Waskiewicz et al., 1999), which is another signalling pathway estradiol may utilise to increase GluN2B, and global protein synthesis, in the OVX female hippocampus. A future experiment to consider would be assessing expression changes of PSD-95, and GluN2B, in the presence of U0126 (MAPK/ERK cascade inhibitor) to see whether ERK is critical in mediating expression changes in OVX females, and males. However, there are other signalling kinases estradiol could also be signalling through.
This chapter highlights that estradiol-mediated increases in PSD-95 and GluN2B expression is via a local protein synthesis mechanism. As a result, there is an increase of PSD-95 and GluN2B at spines and PSD-95 along dendrites. Increased PSD-95 is dependent on mTOR in both sexes whereas, GluN2B is not. This contrasts with the fact that assessment of global protein synthesis indicated that mTOR was not required in the OVX female hippocampus, which could be attributed to the insensitivity to the methods employed in the current study. The data strongly supports that estradiol is able to increase translation along dendrites and within spines and moreover, modulate the distribution of ribosomes.
CHAPTER 6

GENERAL DISCUSSION

SUMMARY OF FINDINGS

Multiple lines of evidence have demonstrated that sex steroid hormones such as estrogens, particularly estradiol, can rapidly regulate cellular and behavioural function. Estradiol can enhance memory acquisition and consolidation within the hippocampus in both male and female rodents in a ‘non-genomic’ manner. The mechanisms underlying estrogenic-facilitation of memory are driven partly through the activation of signalling cascades, resulting in the modulation of synaptic structure and function. These signalling cascades have been implicated in modulating local protein synthesis. Corroborated with evidence that estradiol can rapidly regulate local protein synthesis machinery, it provides a potential role for estradiol to utilise a local protein synthesis mechanism. However, the molecular and cellular mechanisms that underlie estradiol’s ability to regulate local protein synthesis, and whether this is synonymous in both males and females are unknown.

Firstly, the expression profile of key excitatory and inhibitory synaptic proteins was assessed in response to acute estradiol treatment. Estradiol was found to induce a sex-specific change at synapses by regulating the expression of both excitatory and inhibitory proteins within the male and OVX female hippocampus (Chapter 3). This resulted in an increase of several excitatory proteins. This is consistent with previous studies suggesting a shift of the E/I balance towards the excitatory side (Huang and Woolley, 2012; Mukherjee et al., 2017; Oberlander and Woolley, 2016). Additionally, estradiol induced a synonymous change in the excitatory and inhibitory synaptic proteome between the sexes. This suggests that estradiol may regulate synaptic plasticity through different mechanisms within the male and OVX female
hippocampus. A striking observation was that estradiol increased key excitatory proteins such as PSD-95 and GluN2B in both sexes within a time-frame that indicated a non-genomic mechanism was possibly being engaged. Consistent with this, an increase in the rate of protein synthesis was observed in concert with an increase in nascent proteins being produced or targeted to synapses (Chapter 4). Upon investigation, both biochemically and through ICC, estradiol was found to increase both PSD-95 and GluN2B near synapses suggesting estradiol was rapidly enhancing plasticity by orchestrating the increase of these key synaptic proteins (Chapter 5). Critically, data from Chapters 4 and 5 indicate that newly synthesised PSD-95 and GluN2B is being targeted to synapses.

Within this context, one of the main objectives of this thesis was to establish whether estradiol could rapidly regulate protein synthesis in a local protein synthesis manner in the male and female hippocampus. Employing the SUnSET assay in an intact system to address this question, estradiol was demonstrated to increase protein synthesis in male and OVX female hippocampal slices within 2 hours (Chapter 4). This increase was attenuated in the presence of protein synthesis inhibitor suggesting estradiol was using a translation mechanism to induce an increase. This was confirmed when a gene transcription inhibitor had no effect on this increase, suggesting that estradiol may be employing local protein synthesis machinery to mediate this increase. This is consistent with previous studies that have shown estradiol is able to regulate proteins involved in the initiation of translation within 5 minutes (Akama and McEwen, 2003; Fortress et al., 2013; Sarkar et al., 2010). In support, Chapter 5 illustrated increased puromycin incorporation near spines and within dendrites of estradiol treated primary hippocampal neurons. As puromycin incorporation terminates the elongation of amino acid polypeptides, it can be reasoned that there is an increase in active translation within spines and dendrites. Consistent with this, estradiol increased the distribution of ribosomal protein S10 near spines and within dendrites, the same subcellular locations as the site of translation (Chapter 5). This supported the notion that estradiol may be engaging ribosomal machinery to coordinate translation. Amalgamating these findings with the FUNCAT assay to detect newly synthesised proteins, it was observed that nascent proteins were targeted to synapses in estradiol treated neurons, and this was found to be significantly increased in estradiol treated neurons compared to a vehicle control (Chapter 4). Within the
same time-frame, estradiol increased spine density. Notably, dendritic spines with a larger spine area were found to be significantly increased in response to estradiol. Additionally, more nascent proteins were found in larger spines in estradiol treated neurons compared to a vehicle control. Thus, this supports the scenario that the increase in both PSD-95 and GluN2B observed in spines could be dependent on a local protein synthesis mechanism. Therefore, based on these data, it is possible to propose a novel mechanism by which estradiol may be mediating the reorganisation synaptic architecture that may contribute to the memory enhancing effects of estradiol in the male and OVX female hippocampus.

A recurrent theme within the thesis was that estradiol mediated plasticity in a sexually dimorphic manner upon a 2 hour treatment. **Chapter 4** demonstrated that mTOR was required for estradiol to increase global protein synthesis within the male hippocampus but not within the OVX female hippocampus. Critically, it was seen that mTOR was required for estradiol to increase in PSD-95 in both male and OVX female hippocampus and GluN2B only in the males. This highlighted a caveat of using SUnSET to measure global protein synthesis as mTOR may be important in mediating the synthesis of a subset of proteins in the OVX females, such as PSD-95. Additionally, estradiol rapidly phosphorylated ribosomal protein S6 in the males but not OVX females. Therefore, the activation of this ribosomal protein may be important in increasing protein synthesis in the males, but that estradiol could be utilising different signalling molecules within a different pathway in the OVX female hippocampus. Overall, these findings demonstrate that estradiol increased protein synthesis through a local protein synthesis mechanism at synapses through distinct mechanisms in the male and female hippocampus. It was however, beyond the scope of this study to identify which proteins were being synthesised via a local protein synthesis manner following estradiol treatment. However, several lines of evidence from this study would indicate that the synthesis of synaptic proteins would be strong candidates. In the future, studies identifying which proteins are synthesised and moreover, whether they are targeted to and functionally integrated into synapses, will need to be carried out to fully understand the functional impact of this mode of action for estrogen signalling.
The regulation of the synaptic proteome underlies synaptic strength, and thus, also drives synaptic plasticity (Hafner et al., 2018). Indeed, local protein synthesis has been demonstrated to be necessary for synaptic plasticity such as L-LTP (Bradshaw et al., 2003; Vickers et al., 2005), BNDF-induced stimulation (Kang and Schuman, 1995) and mGluR-LTD (Costa-Mattioli et al., 2009). Synaptic turnover is regulated by both local protein synthesis and protein degradation that work in concert (Alvarez-Castelao and Schuman, 2015). Proteins are synthesised and degraded continuously at synapses to modulate cellular function and allow change in response to an internal stimulus such as an action potential or external stimuli such as hormones or induced stimulation (Alvarez-Castelao and Schuman, 2015). These stimuli can locally increase synaptic protein expression either by protein trafficking or local protein synthesis. This provides synapses a rapid local control of their own pool of synaptic proteins to change and modulate its strength in response to these stimuli.

The sexually dimorphic manner by which estradiol reorganises the synaptic proteome of both excitatory and inhibitory synapses suggest that estradiol is having differential effects in both sexes. These differences are important in understanding how estradiol affects synaptic plasticity as this may not be synonymous in each sex. Reiterating the notions of latent effects discussed by Oberlander and Woolley (2016), whereby different molecular mechanisms may underlie the same outcome in both sex, it is essential to consider both sexes in estradiol-related research. Indeed, this was evident within the current study when one signalling pathway did not mediate the increase in global protein synthesis in both sexes. This is particularly important when estradiol may be considered as a therapeutic target, since estradiol does not utilise the same signalling pathway it may mean that estradiol is also activating different ERs as observed by Oberlander and Woolley (2016) in vivo. A caveat to the current literature in estradiol research is that what we currently know about the signalling pathways and ERs mediating synaptic changes and behaviour is derived from OVX females highlighting the need for more research employing male rodent models. Significantly,
many laboratories have started considering using both animal models to investigate estradiol’s effects in the brain. Sexual dimorphism can be observed in a number of diseases therefore, it emphasises the need to consider sex as a variable to tailor sex specific treatments.

A question that arises is that why would estradiol need to engage such a mechanism of local protein synthesis? Estradiol increases dendritic spines within 30 minutes in vitro, which has been shown to be independent of protein synthesis (Srivastava et al., 2008). These novel spines however, are transient unless stimulated. Thus, this led to Srivastava et al. (2008) to propose the ‘two-step wiring plasticity’ model that purports estradiol increases the number of novel spines whilst causing the removal of GluA1-containing AMPARs concomitantly with insertion of GluN1-containing NMDARs into new spines. However, stimulation could lead to the preservation of these nascent spines. Coupled with the trafficking of GluA1-containing AMPAR trafficking into the nascent spines this could result in enhanced synaptic connectivity over a longer period. The data from the current study shows that estradiol not only increased spine area, but also nascent protein within larger spines after 2 hours. Consistent with the ‘two-step wiring plasticity’ model, one possible explanation could be that estradiol-mediated increases in protein synthesis is required to stabilise the nascently formed spines through the synthesis of synaptic proteins specifically at synapses. This could suggest that the initial estradiol-dependent spine formation is independent of protein synthesis, but that nascent spine stabilisation is dependent on local protein synthesis. This would however need to be tested in future studies. But this stabilisation could contribute to the consolidation of memories (Sellers et al., 2015a). Significantly, there has only been one account looking directly at necessity of protein synthesis in the memory enhancing effects of estradiol. Recently, Sheppard et al. (2018) found that a sub-effective dose of anisomycin, but not actinomycin D, inhibited the enhancing effects of estradiol on a rapid paradigm (40 minutes) of social recognition in OVX females. This is the first study to directly consider whether protein synthesis is required for estradiol to increase memory acquisition, but further studies are warranted. Notably, there is an argument that protein synthesis inhibitors such as anisomycin, have been demonstrated to induce gene transcription in vivo, termed ‘gene superinduction’ (Radulovic and Tronson, 2008). The authors explained that decreased protein synthesis may occur within 1-2 hours following injection, but that gene
superinduction extends for several hours following injection. Thereby, it is not clear whether the inhibition could inhibit protein translation. These contradictory notions warrant further research in fully understanding the mechanism by which estradiol-induced protein synthesis may contribute to memory.

The very rapid effects of estradiol such as increase in spine density occur acutely in a protein synthesis independent manner. This is followed by a phase when there is protein synthesis, which may potentially be stabilising estradiol’s earlier effects. Interestingly, we have shown for the first time that estradiol can phosphorylate histone H3 in neurons through the cyto-nuclear shuttling of afadin and subsequent nuclear activation of ERK, which occurs within 30 minutes (Sellers and Watson et al., 2018). Indeed, the phosphorylation of histone H3 is associated with the initiation of gene transcription (Berger, 2007). Thereby, this event likely controls long-lasting changes in gene transcription that may eventually contribute to synaptic function although further testing is warranted before any conclusions can be made. However, it demonstrates that estradiol may be mediating gene transcription in a non-canonical manner. This is in line what is seen with the current study, where estradiol co-ordinates protein synthesis independently of gene transcription with 2 hours. Corroborating all these data, it seems that estradiol may rapidly be inducing changes to synaptic plasticity and neuronal circuitry. This mode of action is important compared to the classical way that estrogens are thought to occur as it gives a rapid control of modulating synapse structure and function thereby, contributing to long-lasting changes in neuronal circuitry.

Relevance to neurological diseases

Abnormal regulation of local protein synthesis has been demonstrated to be a critical factor in the pathophysiology of specific neurodevelopmental and psychiatric disorders (Liu-Yesucevitz et al., 2011; Swanger and Bassell, 2013). This emphasises the importance of gaining a further understanding of the molecular mechanisms controlling the process of protein translation to advocate potential therapeutic targets in the future. One such example is Fragile X Syndrome (FXS), which is characterised by the loss of the fragile X mental retardation protein (FMRP) caused by an expansion of CGG
repeats at the 5’ UTR in the fragile X mental retardation 1 (FMR1) gene (Richter et al., 2015). FMRP is an RNA binding protein (RBP) that reversibly suppresses the translation of specific proteins (Liu-Yesucevitz et al., 2011) such as the group I mGluR mGluR5 receptor and signalling molecules downstream of ERK and PI3/mTOR pathway (Darnell and Klann, 2013). Mouse models of FXS have exhibited elevated levels of protein synthesis in the absence of FMRP. Huber et al. (2002) found exaggerated mGluR-LTD in FMR1 KO mice and Bear et al. (2004) suggested that some symptoms may be due to excessive protein synthesis downstream of group I mGluR activation. Decreased mGluR5 activity has been implicated in alleviating some symptoms in mouse models of FXS and Osterweil et al. (2010) have recently shown that inhibition of mGluR5 reduces protein synthesis. Interestingly, they found that inhibiting ERK also reduced protein synthesis, suggesting that it is the hyperactivity of both mGluR5 and ERK that may contribute to some symptoms of FXS. Following this, Osterweil et al. (2013) found lovastatin, an FDA-approved drug and a Ras-ERK inhibitor, normalised excess protein synthesis in FMR1 KO mice by inhibiting Ras and lowering ERK activity. The treatment with lovastatin is currently being tested in clinical trials. Due to the known safety profile of lovastatin, an open-label study found positive effects of lovastatin after 4 and 12 weeks of treatment in 16 individuals with FXS (Çaku et al., 2014). Recently, lovastatin has been shown to normalise ERK activity in FXS platelets (Pellerin et al., 2016). Thus, lovastatin normalises protein synthesis abnormality, which in turn normalises the synaptic protein or function deficits. Therefore, targeting a protein synthesis pathway or mechanism may be effective in other disorders.

Alternatively, other disorders show synapse loss such as Alzheimer’s disease (Koffie et al., 2011), schizophrenia (Osimo et al., 2018) and major depressive disorders (Kang et al., 2012). In a randomised trial, ketamine was found to produce rapid-acting antidepressant effects (Zarate et al., 2006). Recently, this has been thought to be driven by a local protein synthesis mechanism (Li et al., 2010). Current work within our lab has shown that ketamine increases protein synthesis resulting in the production of synaptic proteins (Creeney et al., under preparation). Ketamine therefore, targets a local protein synthesis mechanism similar to that of estradiol. This could be seen beneficial in disorders like Alzheimer’s disease and schizophrenia where synapse loss is profound. Estrogens seem to be neuroprotective but also neurorestorative and thus,
estradiol’s ability to regulate protein synthesis may result in an increase in synaptic proteins thereby reversing synaptopathies, similar to that of ketamine or lovastatin. Indeed, recent work from our lab have shown that estradiol rapidly rescues spine levels in a cellular model that recapitulates the loss of synapses by manipulating Disrupted in schizophrenia 1 (DISC1) (Erli et al., submitted). However, more research is warranted to test the beneficial effects of estradiol in the context of a local protein synthesis mechanism being therapeutic.

**LIMITATIONS AND FUTURE DIRECTIONS**

**OVX female rodent models**

One of the challenges using intact female rodents to study the influence of estradiol is the naturally fluctuating hormone levels over the estrous cycle that could confound particular behavioural tests (Tuscher et al., 2015). Majority of what is known about the acute effects of estradiol at both cellular and behavioural levels in females *in vivo* has been reported by estrogen replacement in OVX models for this reason. This is particularly true in research investigating the effect of estrogens on memory (Choleris et al., 2012; Frick and Kim, 2018). As with a number of studies within the estradiol research field, the current study utilised OVX females. This approach eliminates circulating estrogens thus, the effect of exogenously applied estrogens can be measured. A caveat to keep in mind is that some androgens are synthesised in the adrenal cortex that may be aromatised into estrogens thereby, contributing to the effects being measured (Srivastava et al., 2013). Additionally, the time between ovariectomy and behavioural or molecular testing could account for variabilities with results within the experiment or across laboratories as hormone deprivation can have confounding effects. In support, studies have illustrated that ovariectomy decreased dendritic spines in the CA1, but not CA3 (Wallace et al., 2006), which has also been previously observed following ovariectomy (Gould et al., 1990). Subjects in Gould et al.’s (1990) study had been assessed for spine loss following 1 week of post ovariectomy and showed less spine loss than those within Wallace et al.’s (2006) study, who were assessed 7 weeks post-ovariectomy. Thus, hormone deprivation begins to alter the synaptic architecture over time (Wallace et al., 2006). This highlights
the importance of keeping the time post ovariectomy the same between animals to ensure consistency over the data. This was maintained within the study where the vendors transported the OVX females 1 week post-ovariectomy and the animals were sacrificed within a 3 days period following 3 days of habituation. A future direction to consider however, would be to assess the changes in global protein synthesis in naturally circulating female mice. This would provide a greater insight into the effect of estrogens in regulating protein synthesis generally.

Alternative methods to investigate local protein synthesis

A limitation to this experimental set up was that estradiol was applied to the whole neuron. Thus, in addition to activating receptors specific for estrogens along dendrites, estradiol would also activate nuclear estrogen receptors. Thereby, the read out of protein translation at dendrites might have some influence from the soma. A number of studies have used severed neuronal dendrites from their cell bodies to specifically investigate local protein synthesis in neurons, without contribution from gene-transcription-dependent translation (Huber et al., 2000; Kang and Schuman, 1996; Tsokas et al., 2005). Critically, dissociating dendrites from their soma compromises the integrity of the neuronal circuitry the neurons have created within a dish (Taylor et al. 2010). Recently Taylor et al. (2010) have developed microfluidic perfusion chambers where single cell neurons are seeded in one part of the chamber and as they develop, the dendrites and axons extend out through microgrooves to another compartment. Through fluidic concentration gradients, the dendrites and axons are maintained in microenvironments allowing local perfusion of dendrites. Recently, FUNCAT has been shown to be coupled with microfluidic chambers to show new protein synthesis at dendrites, without engaging any somatic mechanisms (tom Dieck et al., 2012). A future direction to consider is measuring estradiol-mediated increases in newly synthesised proteins using microfluidic chambers by perfusing estradiol directly at dendrites to eliminate any contribution of somatic activity. Other methods to consider complementing the current data is using approaches that detect mRNAs bound to ribosomes such as the Translating Ribosome Affinity Purification (TRAP) method. This approach labels polyribosomes containing translating mRNAs using a neuronal cell type specific promoter (Heiman et al., 2014; Vlatkovic and Schuman,
2016). This approach allows an in situ profiling of a cell's mRNA translational profile within a specific type of cell (Heiman et al., 2014). Additionally, it would provide spatial information about where actively translating mRNAs can be visualised in estradiol treated neurons. However, the current study was targeted in looking at synaptic proteins. This is a caveat as estradiol has a number of effects within the brain and so a large number of proteins are likely to be synthesised in response. Thus, to appreciate the full extent of the implications of estrogenic-regulation of protein synthesis we need to identify other proteins that are also being regulated within the current time-frame of 2 hours via an unbiased approach using mass spectrometry.

Multiple lines of evidence within the current study provide support for the notion that PSD-95 and GluN2B could be locally synthesised near synapses upon estradiol treatment. An ideal experiment to complement this hypothesis would be to track PSD-95 or GluN2B mRNA translation along dendrites. PSD-95 translation events have previously been tracked at a single molecule level in vitro utilising a venus fluorescent protein tagged to 3' UTR of PSD-95 through live imaging (Ifrim et al., 2015). Another method to consider is to monitor new synthesis of specific proteins is to use time-specific tagging for the age measurement of proteins (TimeSTAMP) (Butko et al., 2012; Lin et al., 2008). The method uses the fusion of the hepatitis C virus protease to an epitope or fluorescent tag within a protein, which can remove itself under basal conditions. In the presence of specific inhibitor however, this removal can be blocked thereby, tagging newly synthesised candidate protein paired with a pharmacological treatment to assess newly synthesised proteins in response to the pharmacological application. Alternatively, Erin Schuman’s laboratory developed a novel approach coupling SUnSET and the proximation ligation assay (PLA; puro-PLA) to visualise specific newly synthesised proteins in situ. PLA detects the spatial coincidence of two antibodies: one that tags newly synthesised proteins (puromycin) and the one that tags a protein target of interest (e.g. PSD-95/GluN2B) (tom Dieck et al., 2015). Newly synthesised proteins are incorporated with puromycin and a puromycin antibody is used to identify them and a second protein specific antibody is used such as PSD-95. Following this, secondary antibodies coupled to different oligonucleotides, PLA{\textsuperscript{plus}} and PLA{\textsuperscript{minus}} are added. When they are in close proximity, a fluorescent signal is obtained via linker oligonucleotides and a ligase creating a ‘rolling circle amplification’ and binding the fluorescently coupled detection probes; this protocol was detailed from tom...
Dieck et al. (2015). This is an advantageous approach to consider in future experiments to identify estradiol mediated newly synthesised PSD-95 and GluN2B specifically at dendrites. Indeed, SUnSET is a fast and easy labelling approach and it would indeed give us in depth information of the site of translation of specific proteins. However, as discussed in Chapter 2, puromycin would lead to premature termination of the labelled polypeptide chain and enhanced degradation of truncated proteins, and this should be considered when attempting puro-PLA. As a substitute, tom Dieck et al. (2015) also proposed that the PLA assay could be paired with FUNCAT (FUNCAT-PLA), which would be a better alternative. The incorporation of AHA would be as detailed in Chapter 2 and due to its mechanism of action, full length proteins would be labelled thereby, avoiding any truncated proteins. Although, the incorporation and labelling is slower compared to SUnSET as it involves an extra step of click chemistry, it is a better alternative when tracking the synthesis of proteins over a few hours, or days (tom Dieck et al., 2015). Overall, these techniques all are excellent considerations to investigate and visualise estradiol mediated translation of PSD-95, GluN2B and any other candidates in the future.

With the knowledge that estradiol may be rapidly mediating the translation of PSD-95 and GluN2B near synapses, other proteins important for synaptic plasticity may also be translated. Alternatively, estradiol may induce the translation of a subset of proteins implicated in diseases. The proteins selected in Chapter 3 were chosen because of their important roles at excitatory and inhibitory synapses. Many other prominent proteins were not looked at that also have a key role at synapses. Therefore, unbiased approaches are warranted to identify target proteins estradiol is translating within 2 hours in both male and OVX female hippocampus.

Other signalling cascades implicated in estradiol-mediated protein synthesis

A pathway that has been investigated and implicated in estradiol research that may contribute to the estradiol-mediated global protein synthesis in OVX females is the PI3K/Akt pathway. Estradiol phosphorylates Akt within 5-15 minutes in vitro and in vivo (Akama & McEwen 2003; Briz & Baudry 2014; Fan et al. 2010; Sellers et al. 2015b) and activates ERK through Akt in the dorsal hippocampus (Fan et al., 2010). This
demonstrates a complex cross-talk between these kinases. The Akt pathway also leads to activation of mTOR (Li et al., 2004) but can increase phosphorylation of 4EBP1 directly as well (Gingras et al., 1998). Fan et al. (2013) proposed a signalling model which suggested that estadiol activates PI3K, which activates ERK directly or indirectly via Akt suggesting a close relationship between these two signalling pathways. Akt has been reported to be necessary, alongside ERK and mTOR, for enhancing OR and OP memory tasks in response to estradiol in OVX female rodents (Fortress et al., 2013). But it is not clear whether these kinases depend on each other, cross-talk with each other, or even regulate the same processes in response to estradiol. Nevertheless, it is possible that Akt and ERK may be converging in increasing protein synthesis in OVX females in response to estradiol. An important experiment to conduct would be to inhibit both ERK and Akt pathways to see if either or both are required to increase estradiol-mediated global protein synthesis.

Role of ERs

A subsequent step to fully understand estradiol's mediation of local protein synthesis would be to assess which receptor is driving this increase. Particularly, it would be interesting to see whether the same receptor mediates these effects of estradiol in both sexes. Nevertheless, the scope of this study was to consider whether estradiol induces protein synthesis in a local protein synthesis dependent manner. Future studies are warranted to investigate which ERs are important for this effect. Additionally, a caveat to the current study is that a bath application of estradiol was used and so multiple ERs and other receptors could also be activated. Possibly, one ER is specifically driving the effects on protein synthesis which can be determined in vivo as there is a tight regulation of estradiol signalling thus, protein synthesis could be occurring only at select synapses in select circuits. However, having the type of diversity that may occur in the current experimental set up may allow for a number of cellular outcomes.
Estrogenic signalling within the cerebral cortex

There is mounting evidence on the role of estrogens within the hippocampus and has been the focus of many studies. However, work from our lab has exhibited an important role of estradiol signalling within the cerebral cortex (reviewed in Sellers et al. 2015a). ERs are also present in the cortex (Almey et al., 2015; Milner et al., 2001, 2005) and have been localised to dendrites in vitro (Sellers et al. 2015b) in addition to the presence of aromatase (MacLusky et al., 1994; Yague et al., 2006) at synapses (Srivastava et al., 2010). Indeed, there are some similarities in the cellular effects of estradiol within the hippocampus to that of the cortex for example, rapid increase in dendritic spines (Sellers et al., 2015b; Srivastava et al., 2008, 2010). However, the underlying molecular mechanisms mediating estrogen’s actions within the cortex may be distinct to that of hippocampus. Preliminary in vitro results from the current study investigated estradiol’s effect on global protein synthesis following 2 hour treatment biochemically in primary cortical neurons and prefrontal cortex slices from male and OVX female mice. The results were synonymous to the results reported in this study. However, more experiments to this depth are needed to decipher the signalling pathways needed to increase protein synthesis and whether it is dependent on a local protein synthesis mechanism.

CONCLUDING REMARKS

This study highlighted the importance of considering both sexes when investigating molecular mechanisms underlying a cellular process. Sex differences have been found in many prominent diseases including AD and schizophrenia. Therefore, considering sex as a variable is of importance to tailor treatments to each sex. This study is first of its kind to directly demonstrate that estradiol mediates an increase in protein synthesis, in a local protein translation manner, in both male and OVX female hippocampus. It proposes a novel mode of estrogen signaling that could result in long-lasting changes in neural circuitry and cognitive function. Albeit more studies are warranted to identify what proteins are being rapidly synthesised, which could aid in recognising potential therapeutic drug targets.
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Appendix 1  Puromycin antibody validation for Surface Sensing of Translation (SUnSET) assay. A-C, In the absence of puromycin treatment, the puromycin antibody did not bind to any signal in DIV 24 primary cortical neurons (A+B) and male acute hippocampal slices (B). This signal was reduced in the presence of protein synthesis inhibitor, anisomycin (A). This was validated both through western blotting for SUnSET-WB (A-B) and immunocytochemistry for SUnSET-ICC (C). Scale bar = 50 µm.
Appendix 2  *Time course for mTOR activation over 2 hour estradiol treatment.*

A+B, Representative western blot and quantification of DIV 25 primary cortical neurons temporally treated with 10 nM estradiol. Estradiol rapidly activated mTOR with 30 minutes, and this persisted until 60 minutes; n=6 per condition. One-way ANOVA, Bonferroni corrected; Error bars represent mean ± SEM; ** p = <0.01.
Appendix 3 Validation of Fluorescent noncanonical amino acid tagging (FUNCAT) assay. A+B, Representative 20x (A) and 40x (B) confocal images of primary cortical (A) and hippocampal (B) neurons. A Pseudocolor lookup table indicated higher AHA-tagged newly synthesised proteins along dendrites after 2 hour 4 mM AHA treatment compared to 30 minutes. B In the absence of AHA, the Alexa Fluor 555-Alkyne tag does not fluoresce any signal. The signal is decreased in the presence of protein synthesis inhibitor, anisomycin. Scale bar = 100 µm (A) and 50 µm (B).