The Effects of Estrogen Treatment on Neurocognition in Healthy Young Women and Women with Schizophrenia

by

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BA (Psych/Psychophys), BAppSci Honours (Psychophys)

A thesis submitted for the degree of

Doctor of Philosophy
(Clinical Neuropsychopharmacology)

Brain Sciences Institute
Swinburne University of Technology
Melbourne, Australia 2008

Abstract

The wealth of biochemical, molecular and behavioural evidence to support estrogen, in particular 'estradiol' (E_2), as a neuroprotective agent in the brain has lead to the proposal of estrogens as possible treatment for the cognitive deficits inherent to various neurological and mental disorders, such as schizophrenia. Despite this, the mechanisms underlying E_2's actions in the brain are largely unknown, although the cholinergic system shows promise as a key modulator of E_2 effects, given the prominent role of this neurotransmitter system in fundamental cognitive processes. Therefore the aim of this thesis was to investigate the cognitive effects of one month of 100µg/day transdermal E_2 treatment in healthy young women (Experiment One) and women of child-bearing age with schizophrenia (Experiment Two). In addition, the role of the cholinergic system in mediating the cognitive effects of estrogen was also explored in our sample of healthy young women. Both experiments were placebo-controlled randomized double-blind designs and examined the following cognitive domains: declarative verbal memory and learning, verbal fluency, working/visual memory, attention, cognitive flexibility and information processing/psychomotor speed.

Short-term E_2 treatment in healthy young women had selective positive effects on delayed verbal recall and spatial working memory, however the majority of cognitive domains were unaffected. Furthermore, overall E_2 treatment did not protect against (or attenuate) the cognitive deficits induced by the muscarinic receptor antagonist scopolamine. In addition, one month E_2 treatment did not improve cognitive functioning for our sample of women with schizophrenia. These findings add to the already largely inconsistent literature and highlight the complex effects of E_2 treatment on cognitive function, which may be further influenced by age, endogenous estrogen levels and duration of treatment. These findings suggest that adjunctive E_2 treatment may not be an effective treatment for cognitive deficits in women of child-bearing age with schizophrenia. However, given these are two of the few studies to have investigated the cognitive effects of E_2 treatment in this age

cohort, further research is required before definitive conclusions can be drawn. Future research should investigate the cognitive effects of E_2 in post-menopausal women with schizophrenia and focus on alternative means for improving cognitive deficits. Further investigation into the underlying neurochemical mechanisms of estrogen in the human brain is also needed to help determine the nature of estrogen's variable effects on cognitive function and it's role in the pathophysiology of schizophrenia and related disorders.

Acknowledgements

I would firstly like to thank the Australia Rotary Health Research Fund for awarding me the Ian Scott Fellowship, it is an honor to be affiliated with such a highly regarded organization and I greatly appreciate the support they provided me throughout the years of my PhD.

There are also a number of people that I would like to acknowledge and thank for their support and assistance during this PhD. My sincerest thanks go to my coordinating supervisor Associate Professor Pradeep Nathan, whose enthusiasm for psychopharmacology and well-controlled scientific research was inspiring and persuaded me to take-up a PhD in this field. I am extremely grateful for his guidance, encouragement and honest advice throughout the years, and despite our disagreements I am glad we always managed to find a common ground and preserve our friendship. I would also like to thank my secondary supervisor Professor Jayashri Kulkarni for introducing me to the world of psychiatry research, a field I am excited to pursue further in my academic career.

I would like to express my great appreciation to Professor Rodney Croft who advised me on all statistical analytical methods, and who provided me with the same support and time that he affords to his own PhD students. I would also like to thank Dr. Susan Illic who performed medical examinations and all the nurses from the School of Integrative Medicine who helped with administering injections, in particular Susan Vitetta who kindly gave up her free time on her days off.

I am extremely grateful to my colleagues from the Brain Sciences Institute and my dearest friends for their words of wisdom, enduring support and encouragement, especially Danijela Piskulic, Valérie Guille and Victoria Nicoloudis, who also kindly proof read my thesis chapters in a timely manner.

I wish to express my deep gratitude to my parents Laurel and Rudy, and to my brother Cory, for their love, support and their understanding which has never faulted throughout the years. Special thanks also go to my uncles Rafer and Graeme for

their genuine interest and enthusiasm when discussing my PhD topic, as it was these little things that motivated me to soldier on during the hard times.

Lastly, I would like to dedicate this PhD thesis to my grandfather, Trevor Gordon, who always taught me to follow my dreams and to wholeheartedly embrace the challenges life has to offer.

Declaration

I declare that this thesis does not incorporate, without written acknowledgement, any material that has previously been submitted for the award of any other higher degree or diploma in any university, college, or other educational institution; and to the best of my knowledge, this thesis does not contain any material previously published or written by another person except where due reference is made in the text of the thesis.

I declare that the ethical principles and procedures specified in the Swinburne University of Technology Human Research Ethics document on human research and experimentation have been adhered to in the presentation of this thesis.

Name: Cali Bartholomeusz

Signed: _____

Date: _____

List of Publications

The following is a list of publications arising from the research projects contained in, or run in conjunction with the current thesis.

Peer-Reviewed Journal Publications

Kulkarni J, DeCastella A, Fitzgerald PB, Gurvich C, Bailey M, **Bartholomeusz C**, Burger H (2008): Estrogen - a potential new treatment approach for severe mental illness. *Arch Gen Psychiatry 65*(8):955-960.

Bartholomeusz C, Wesnes K, Kulkarni J, Croft J, Vitetta L, Nathan P.J (2008): Estradiol treatment and its interaction with the cholinergic system: effects on cognitive function in healthy young women. *Horm Behav 54*(5):684-693.

Published Conference Abstracts

Kulkarni J, **Bartholomeusz C**, White S, De Castella A, Nathan PJ, Fitzgerald P, Burger H (2004). Estradiol as an adjunct to antipsychotics: A possible treatment for women with schizophrenia. *Int J Neuropsychopharmacol 7*, Suppl 1, June 2004:S423

Bartholomeusz CF, Kulkarni J, Wesnes KA, Vitetta L, Croft RJ, Nathan PJ (2006). Are the effects of estrogen on cognition mediated via modulation of the cholinergic system? *Int J Neuropsychopharmacol 9*, Suppl 1, July 2006:S122

Kulkarni J, **Bartholomeusz CF**, Sheppard S, White S, DeCastella A, Fitzgerald P, Nathan PJ (2006). The effects of adjunctive estradiol on cognition performance in women with schizophrenia. *Int J Neuropsychopharmacol 9*, Suppl 1, July 2006:S154

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List of Abbreviations

[^{123}I]IBVM	[^{123}I] Iodobenzovesamicol
^{125}I-estrogen	17α-iodovinyl-11β-methoxyestradiol
3MSE	Modified Mini Mental State Examination
5-HT	5-Hydroxytryptamine (also called *serotonin*)
A	Androstenedione
Aβ	β- Amyloid
AC	Adenylyl Cyclase
ACh	Acetylcholine
AChE	Acetylcholine Esterase
AChEI	Acetylcholine Esterase Inhibitor
AD	Alzheimer's Disease
AF-1	Activation-Function-1
AF-2	Activation-Function-2
AIMS	Abnormal Involuntary Movements Scale
Akt	*see PKB*
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
acid	
ANOVA	Analyses of Variance
AP-1	Activation Protein-1
ApoE	Apolipoprotein E
BDNF	Brain-Derived-Neurotrophic Factor
BF	Basal Forebrain
BMI	Body Mass Index
BOLD	Blood-Oxygen-Level-Dependent
BSI	Brain Sciences Institute
BSO	Buthionine Sulfoximine
BSRT	Buschke Selective Reminding Task
BVRT	Benton Visual Retention Test
Ca^{2+}	Calcium
CAMCOG	Cambridge Cognitive Examination for Mental Disorders of the Elderly

cAMP	Cyclic Adenosine Monophosphate
CBT	Cognitive Behavioural Therapy
CDR	Cognitive Drug Research
CEE	Conjugated Equine Estrogens
CFF	Critical Flicker Fusion
cGMP	Cyclic Guanosine Monophosphate
ChAT	Choline Acetyltransferase
ChAT-IR	Choline Acetyltransferase Immunoreactivity
CNS	Central Nervous System
COWAT	Controlled Oral Word Association Test
CR	Cognitive Remediation (Therapy)
CRE	cAMP Response Element
CREB	cAMP Response-Element Binding-Protein
CRT	Choice Reaction Time
CVLT	California Verbal Learning Test
dbB	Diagonal Band of Broca
DBD	(DNA) Binding Domain
DHEA	Dehydroepiandrosterone
DLPFC	Dorso-lateral Prefrontal Cortex
DNA	Deoxyribonucleic Acid
DMP	Delayed Matching-to-Position
DNMP	Delayed Non-Matching-to-Position
DOPAC	3,4-dihydroxyphenylalanine
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition
DSST	Digit Symbol Substitution Test
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
EB	E_2 Benzoate
EDP	E_2 Dipropionate
EE	Ethinyl Estradiol
EEG	Electroencephalogram

EPT	Estrogen-Progesterone Treatment
EPSPs	Excitatory Postsynaptic Potentials
ER	Estrogen Receptor(s)
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
ER-γ	Estrogen Receptor gamma
ERE	Estrogen Response Element
ERK	Extra-Cellular Signal-Regulated Kinase
ERP	Event-related Potential
ET	Estrogen only Treatment
$FeCl_2$	Ferrous Chloride
$FeSO_4$	Ferrous Sulfate
fMRI	Functional Magnetic Resonance Imaging
FSH	Follicle Stimulating Hormone
GABA	γ-Aminobutyric Acid
GAD	Glutamate Decarbolaze
GnRH	Gonadatropin Releasing Hormone
H_2O_2	Hydrogen Peroxide
HACU	High-Affinity Choline Uptake
HDL-C	High-Density Lipoprotein Cholesterol
HPA	Hypo-thalamo-pituitary
HT	Hormone Treatment
[I-123]IQNB	Iodinated Quinuclidinyl Benzilate
IGF-1	Insulin-like Growth Factor 1
i.m	Intramuscular
IQ	Intelligence Quotient
KO	Knock Out (mice)
LBD	Ligand-Binding Domain
LH	Luteinising Hormone
LTP	Long-Term Potentiation
M	Mean
MAPK	Mitogen-Activated Protein Kinase
MATRICS	Measurement and Treatment Research to Improve

	Cognition in Schizophrenia (Initiative)
MCC	Methylcarbamylcholine
MMSE	Mini Mental State Examination
MORE	Multiple Outcomes of Raloxifene Evaluation (study)
MPA	Medroxy-Progesterone Acetate
MPTP	1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine
mRNA	messenger Ribonucleic Acid
MS	Medial Septum
N	Number in sample
NART	National Adult Reading Test
NBM	Nucleus Basalis Magnocellularis
NF-κB	Nuclear Factor kappa-B
NIMH	National Institute of Mental Health
NMDA	N-methyl-D-aspartate
nmol/L	Nanomoles per litre
NS	Non-significant
OVX	Ovariectomized
P	Progesterone
PANSS	Positive and Negative Syndrome Scale
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
PI3K	Phosphatidylinositol-3-Kinase
pmol/L	Picomoles per litre
PKA	Protein Kinase A
PKB	Protein Kinase B (*also called Akt*)
PKC	Protein Kinase C
RAM	Radial Arm Maze
RAVLT	Rey Auditory Verbal Learning Test
rCBF	Regional Cerebral Blood Flow
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RT	Reaction Time
SAP	192 IgG-saporin

sβAPPα	Soluble fragment of the Aβ Precursor Protein
SCIT	Social Cognition and Interaction Training
SD	Standard Deviation
SEM	Standard Error of the Mean
SERMs	Selective Estrogen Receptor Modulators
SHBG	Sex Hormone-Binding Globulin
SOPHIA	Soy and Postmenopausal Health in Aging (study)
SPECT	Single Photon Emission Computed Tomography
SPSS	Statistical Package for the Social Sciences
SRF	Serum Response Factor
SRT	Simple Reaction Time
T	Testosterone
TMT	Trail Making Test
U/L	Units per litre
VAChT	Vesicular Acetylcholine Transporter
VAMS	Visual Analogue Mood Scale
WAIS	Wechsler Adult Intelligence Scale
WAIS-R	Wechsler Adult Intelligence Scale- Revised
WHI	Women's Health Initiative (study)
WHIMS	Women's Health Initiative Memory Study
WMS	Wechsler Memory Scale
WMS-III	Wechsler Memory Scale – Third Edition

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SECTION ONE:
ESTROGEN: FROM BIOCHEMISTRY TO PHYSIOLOGY

Chapter 1

Biochemical Basis of Estrogens & their Interactions with Neurochemical, Cellular & Molecular Systems in the Brain

1.1 General Introduction

The female gonadal hormones have numerous functions throughout the lifespan, including sexual differentiation, regulation of gonadotrophins, regulation of prolactin secretion, and modulation of sexual motivation and behaviour. However, it's the non-reproductive mechanisms of estrogen that have long been the focus of research since the discovery of diverse estrogenic effects on breast and endometrial tissue (Flototto et al 2001), vascular endothelium and aortic smooth muscle (Farhat et al 1996), and osteoblasts and chondrocytes of bone (Kusec et al 1998). Much attention has been given to these areas of research due to their implications in breast and endometrial cancers, heart disease and osteoperosis, respectively. However, a great amount of attention has also fallen on estrogen's neuroprotective effects in the brain and central nervous system (CNS). While Pfaff and Keiner (1973) were the first to discover estradiol in the hypothalamus and amygdala of the mammalian brain it was not until the late 80s that estrogen was recognized as having a potential role in brain regions which were unrelated to neuroendocrine functioning (Loy et al 1988). The hippocampus was the initial focus of autoradiography and immunocytochemistry labeling of estrogen receptors (ERs), and sparked much excitement in the neuroscience field. Since then a second ER isoform has been discovered and both ER subtypes have been found located in numerous brain regions in addition to the hippocampus. These include: the midbrain, brain stem, pre-optic area, spinal cord, septum, and cerebral cortex (Pelletier 2000).

In conjunction with the discovery of estrogen receptors in the brain was the exploration and discovery of estrogen's 'neuroprotective mechanisms'. The most potent of estrogens, estradiol, has been shown to: protect from oxidative stress and β-amyloid toxicity, promote neurogenesis, modulate neuronal excitability, and interact with various neurotransmitter systems including the cholinergic, dopaminergic and serotinergic systems, all of which are essential for cognitive functioning (Garcia-Segura et al 2001). The implications these finding have on age-related cognitive decline, as well as neurodegenerative and neurological disorders including mental illness, are widespread and have lead to a growing appreciation for and interest in estrogen's multifarious actions in the brain. The focus of this thesis is on the

neurocognitive effects of estradiol, the possible role of the cholinergic system in mediating these effects and the implications of such effects on cognitive function in women with schizophrenia.

This thesis is divided into three sections. Section One (*Estrogen: From Physiology to Biochemistry*) contains the current chapter which first outlines the natural fluctuations of estrogens in relation to the female reproductive cycle and gives an overview of the biochemical and pharmacological basis of estrogens and their receptors. This chapter also presents a review of the neuroprotective mechanisms of estrogens and their interactions with various neurochemical systems in the brain. Section Two (*Estrogen, Cognition and the Cholinergic System*) begins with Chapter 2 and consists of a review and critique of both animal and human research with a focus on the behavioral effects of estrogen on cognition. Chapter 3 then gives an overview of the effects of estrogen on the cholinergic system and the implications this has for cognitive functioning. This leads into Chapter 4: the rationale and the thesis objectives. Also within Section Two is Chapter 5, the first of two experimental studies, which involved healthy control participants who underwent a cholinergic drug challenge after receiving estradiol treatment for four weeks. This study investigated the effects of estradiol treatment on cognitive function and tested the hypothesis that estradiol exerts it's neurocognitive effects via modulation of the cholinergic system. The third section (*Cognitive Dysfunction in Schizophrenia: Exploring Estrogen as an Adjunctive Treatment Option*) contains Chapter 6, which begins with a brief review of cognitive functioning in schizophrenia and the current treatment options available for improving cognitive deficits in this clinical cohort. Chapter 6 also comprises the second experimental study, which involved women with schizophrenia who underwent estradiol treatment for four weeks. This study examined the usefulness of estradiol treatment as an adjunct to antipsychotic treatment in the alleviation of cognitive deficits for this sample population. Lastly, Section Four (*General Discussion and Conclusions*) comprises Chapter 7, which discusses the implications of the current findings, explores the risks associated with estrogen treatment and concludes with proposed directions for future research.

1.2 Estrogen and the Reproductive Cycle

It is important to briefly reflect on the natural variations in endogenous estrogens within the reproductive cycle. This has been used as a non-invasive, albeit sometimes problematic method of investigating the effects of estrogen on cognitive processes as well as mood and behavior. The duration of the reproductive cycle can vary considerably from woman to woman, which can be due to not only pharmacological agents but numerous lifestyle factors including weight, level of exercise, eating habits and stress (Carpenter 1994; Castelo-Branco et al 2006; Williams 2003). Onset of the reproductive years usually begins around the age of 9-12 and ends around 45-55, when the ovaries stop producing ova, menstruation ceases and estrogen levels decline (known as the menopause). For non-pregnant, post-menache and pre-menopausal women the reproductive cycle lasts approximately 24-35 days, but is theoretically and ideally a 28-day cycle (Tortora and Grabowski 1996). The reproductive cycle is regulated by the endocrine and ovarian hormones and consists of both the 'menstrual' and 'ovarian' cycles, which run synergistically to prepare the body for possible fertilization. The reproductive cycle is broken into two phases, the follicular phase and the luteal phase, which occur either side of ovulation (see Figure 1 for depiction of changes occurring in the ovarian and uterine cycles).

The beginning of the follicular phase (first day of menstruation) is characterized by the enlarging of ovarian follicles, comprising thecal and granulosa cells which grow due to the presence of Follicle Stimulating Hormone (FSH). This hormone is released from the anterior pituitary gland, which is in turn controlled by the release of gonadatropin releasing hormone (GnRH) from the hypothalamus. The hypothalamic release of GnRH to the anterior pituitary is governed by biofeedback mechanisms with estrogen and progesterone, which are low during menstruation (see Figure 1 for variations in the levels of ovarian and adenohypophysial gonadatropins). FSH also has the role of initially stimulating the secretion of estrogen from the growing ovarian follicles during the early phase of the cycle (Tortora and Grabowski 1996).

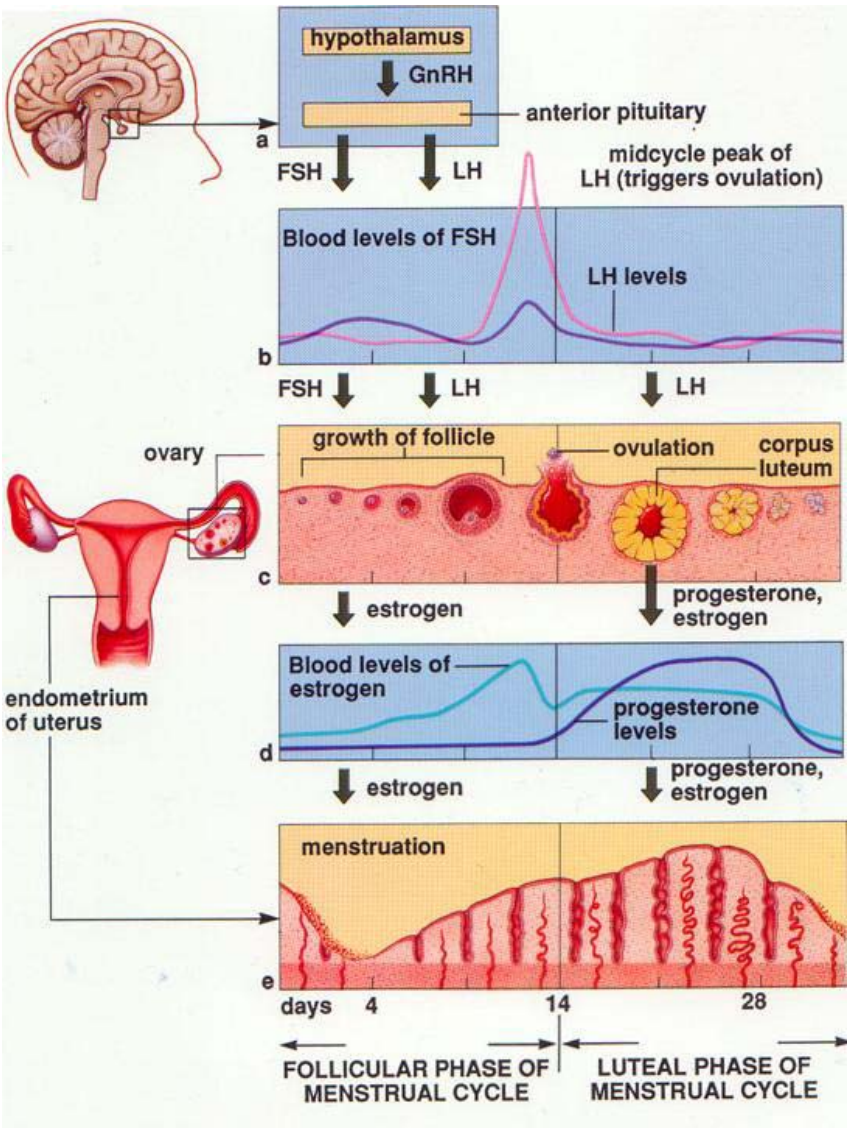

Figure 1. The Female Reproductive Cycle.
The female reproductive cycle consists of the 'menstrual' and 'ovarian cycles'. a) release of hypothalamo-pituitary hormones are triggered via biofeedback with ovarian hormones b) the LH surge triggers ovulation c) the ovarian follicle continues to grow until ovulation d) the rise in estrogen levels just prior to ovulation triggers the LH surge e) the endometrium wall thickens for the possible imbedding of the fertilized ova, which is subsequently shed if fertilization does not occur. [Note]

[Note] Figure is from: http://faculty.sunydutchess.edu/Scala/Bio102/PDF/Menstrual.jpg, last viewed on 4th June 2007

The other adenohypophysial gonadatropin prominent during the follicular phase, is luteinising hormone (LH). This hormone stimulates thecal cells to synthesize androgens, namely androstenedione, the main precursor of androgens and estrogens. At the same time FSH stimulates the aromatizing enzyme in the granulosa cells to convert androgens to estrogens (predominantly 17β-estradiol; E_2). Due to the increasing amount of E_2 during the mid-follicular phase, the dominant ovarian follicle continues to proliferate via a positive feedback effect of E_2. During this time E_2 continues to be secreted along with the protein heterodimer hormone 'inhibin' (Laycock and Wise 1996). Around day 9 of the cycle E_2 has a selective negative feedback effect on FSH, while continuing to allow the synthesis of LH receptors and the further proliferation of the ovarian follicle. The E_2 level continues to rise and once maintained at this high level for at least 36 hours it exerts a positive feedback effect on the hypothalamus and pituitary. This causes a sudden surge in LH, resulting in ovulation and the end of the follicular phase.

The surge in LH is accompanied by a smaller surge in FSH. The LH surge also triggers the granulosa cells to then synthesize androgens into progestogens (predominantly 17 α-hydroxyprogesterone) rather than estrogens (Laycock and Wise 1996). After ovulation the LH surge converts the remaining follicular cells (left over from ovulation) into a corpus luteum, which is predominantly made up of hypertrophied granulose cells. The luteal phase involves a significant increase in the production of progesterone, which causes a small increase in basal body temperature, which can be used as an external marker of this stage in the menstrual cycle. This progressive rise in progesterone is accompanied by a smaller increase in E_2. When at their peak, around the mid-luteal phase (day 20-22), progesterone predominantly initiates a negative biofeedback effect on the hypothalamo-hypophysial axis. In conjunction with the negative biofeedback effect of E_2 and inhibin, the adenohypophysial gonadotropins are withdrawn and ovarian steroid production decreases to the point where luteolysis occurs (degeneration of the corpus luteum). This coupled with the onset of menstruation marks the end of the luteal phase (see Figure 2 for summary of physiological effects of the ovarian hormones).

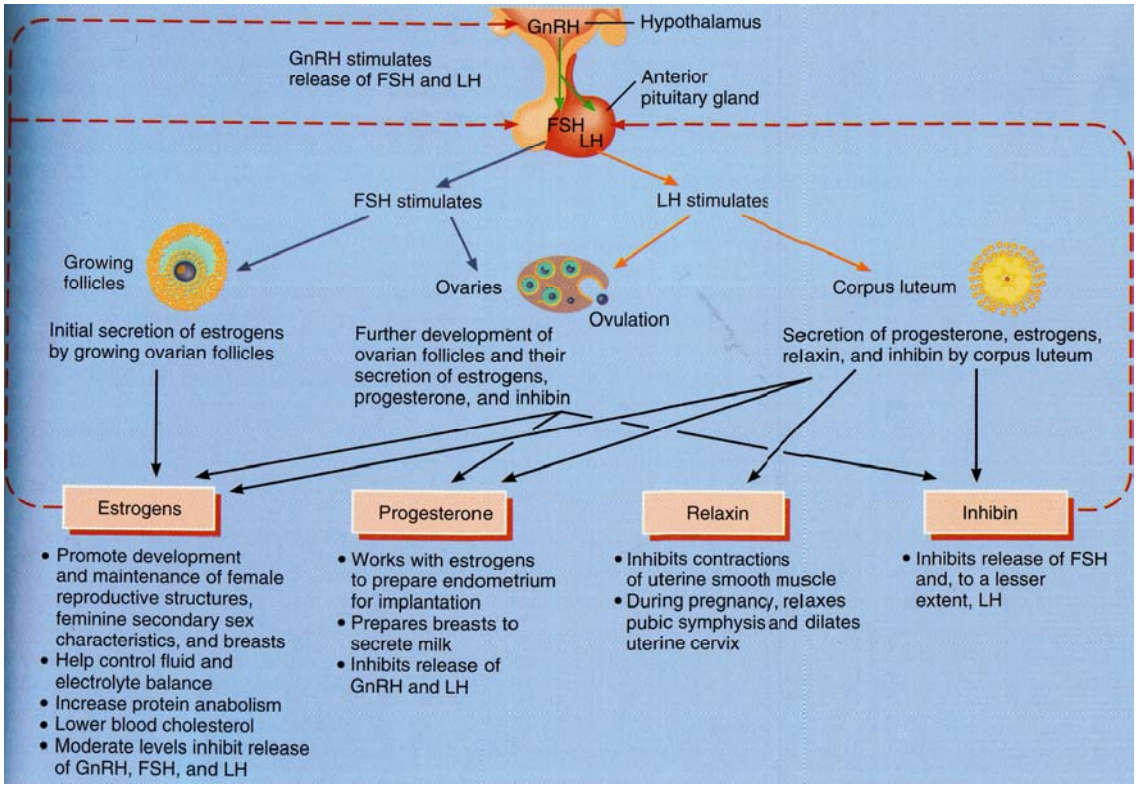

Figure 2. Physiological Effects of Ovarian Hormones (*adopted from Tortora and Graowski 1996, p937)*.

Using the menstrual cycle as a method of linking a causal effect of estrogens to cognition can be difficult as there are other hormones involved that may themselves influence cognitive processes, such as progesterone (Singh 2006). In addition, the regulation of endogenous estrogens is complex and a number of other molecules play a role in the reproductive cycle including oxytocin, vasopressin, catecholamines, prostaglandins and growth factors (Laycock and Wise 1996). As mentioned earlier the menstrual cycle does not always last for exactly 28 days. The length can fluctuate from one month to the next, thus it has been important to measure blood hormone levels when using the menstrual cycle phase as a methodological construct. Having said this however, serum/plasma E_2 levels have recently been suggested to no longer be a reliable indication of the levels in brain regions capable of producing their own estrogens locally, due to the tissue-specific regulation of aromatase expression (Simpson 2003). In addition, the average ranges of hormone levels during particular phases of the cycle in healthy women are generally quite large,

further testifying to the variability between women (see Table 1 for normal hormone ranges for men and women).

Table 1. Reference Ranges for Normal Hormone Levels for Females and Males*

	Ovarian Cycle Phase			Postmenopausal	Male
Hormone	Follicular	Mid-cycle	Luteal		
Estradiol (pmol/L)	143-694	345-1864	176-1134	<150	<283
FSH (U/L)	4-13	5-22	2-13	>20	1-8
LH (U/L)	1-18	24-105	0.4-20	>15	2-12
Progesterone (nmol/L)	0.5-4.5	15.9-63.6	10.6-81.3	<2.3	0.9-3.9
Testosterone (nmol/L)	<3.8	<3.8	<3.8	<3.8	6.9-28.1

* reference ranges are approximate serum levels *(Leonard 2004)*.

1.3 Pharmacological and Biochemical Basis of Estrogens

1.3.1 Synthesis, Storage and Metabolism

The term ‘estrogen’ is used loosely to refer to one or a composite of different types of synthetic and endogenous sex hormones and their metabolites. Thus, the terms ‘estrogen’ and ‘estrogens’ are often used interchangeably. There are three naturally occurring estrogens produced by the female body. The most potent of these is E_2. The other two natural estrogens are estrone (E_1) and estriol (E_3). Estriol is a weak estrogen derived from E_1 and is found in smaller quantities than either E_2 or E_1, other than during pregnancy (Gruber et al 2002). Estrogens share a similar chemical structure and consist of four rings of carbon atoms (see Figure 3 for chemical structure and binding potency of the three endogenous estrogen hormones). Estrogens are 18 carbon steroids, have a low molecular weight and are lipophilic,

thus they can easily cross their target cell membrane and the blood-brain barrier without requiring aided transport. In adult females, after synthesis approximately 40% of E_2 binds to sex hormone-binding globulin (SHBG), while approximately 58% binds to serum albumin, leaving approximately 1-2% free (Notelovitz 2006).

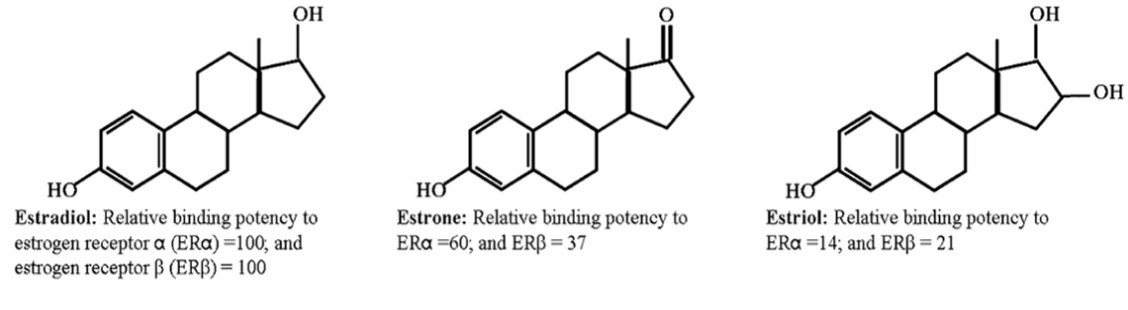

Figure 3. Chemical Structure of Endogenous Estrogens and Relative Binding Potencies *(adopted from Gleason et al 2005, p96).*

Estrogens, and steroids alike, are derived from cholesterol. Before estrogens can be synthesized, hydrolysis of cholesterol esters and the uptake of cholesterol into mitochondria must first take place in the cells of the target organ. There, the enzyme complex 'cholesterol desmolase' hydrolates cholesterol into pregnenolone via the enzyme aromatase cytochrome P450, which is encoded by the CYP19 gene localised on chromosome 15q21.2 (Bulun et al 2003). Pregnenolone is then converted to progesterone through dehydrogenation and double-bond isomerization, or to 17α–OH-pregnenolone (Mathews and Van Holde 1996). Progesterone is then converted to 17α-OH-progesterone which in turn is converted to androstenedione. E_2 can be synthesized from either testosterone or E_1 (see Figure 4 for principle synthesis and conversion pathways for androgens and estrogens). The rate of steroid synthesis is controlled by the hypothalamus and anterior pituitary (as depicted earlier). Because steroid hormones are not stored after synthesis, the rate of synthesis correlates with circulating plasma hormone levels (Mathews and Van Holde 1996).

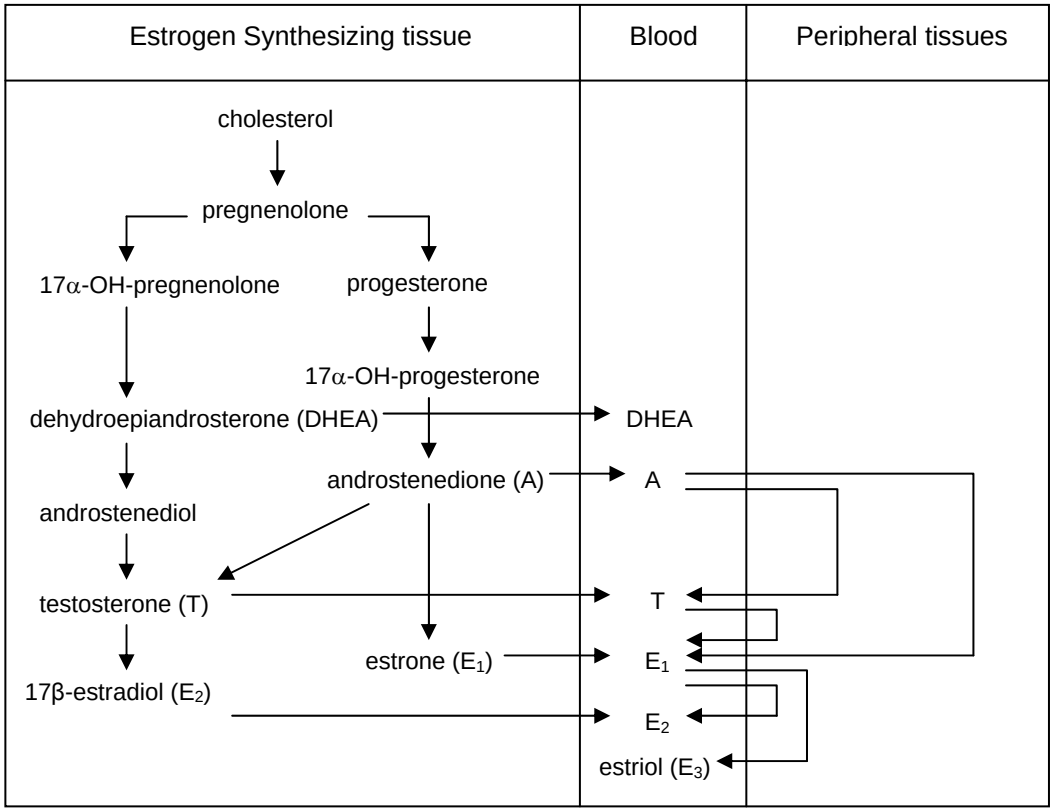

Figure 4. Principle Synthesis and Conversion Pathways for Androgens and Estrogens *(adapted from Laycock & Wise 1996, p171).*

Estrogens are primarily produced by granulosa cells (and to some degree thecal cells) in the ovaries, and to a lesser extent the corpus luteum, the fetoplacental unit during pregnancy and the adrenal glands (Laycock and Wise 1996). As stated earlier, estrogens have more recently been discovered in non-reproductive tissues where they act as paracrine or possibly intracrine factors at the sites locally (Simpson 2003). Estrogens are also produced by the male gonads where testosterone and androstenedione are converted to E_2 and E_1 respectively. In women, apart from the ovaries, adipose tissue is the second most common site of estrogen production and is an alternative source of estrogens for postmenopausal women, albeit limited and dependant on the woman's percentage of body fat. Metabolism of estrogens occurs partially in the gut but mostly in the liver through sulfation or glucuronidation, the conjugates of which are excreted into the bile or urine by the kidneys (Gruber et al 2002). Conjugates passing through the intestinal tract can be hydrolyzed by intestinal flora and thus reabsorbed into the bloodstream, causing an enterohepatic

circulation. Estrogens are also metabolized by hydroxylation and methylation to create catechol and methoxylated estrogens, some of which are considered to be carcinogenic (Gruber et al 2002).

1.3.2 Synthetic Estrogens

A large number of commercially produced estrogens and antiestrogens are on the market and are used for the purpose of contraception, hormone therapy (HT) after the menopause (HT refers to either: estrogen only treatment [ET] or estrogen-progesterone treatment [EPT]), and the treatment of osteoperosis, breast cancer and endocrine disorders. These estrogen preparations are administered via various routes including oral, transdermal, topical, vaginal, subcutaneous and intramuscular. The estrogens used in these preparations can generally be categorized into four types: esterified estrogens, conjugated equine estrogens (CEE), ethinylestradiol and E_2 (Ruggiero and Likis 2002). There is some discrepancy within the literature as to which of these manufactured estrogens can be classified as 'natural'. However, according to basic biology E_2 is really the only natural form available on the market, given it is a single compound and resembles the only estrogen naturally produced by the female body (Notelovitz 2006). Despite this, because manufactured E_2 is synthesized from the nonestrogenic steroid precursor 'diosgenin' (found in plant sterols such as soybeans, or white Mexican yams), some argue that it is synthetic. Conversely some people believe CEEs are the only natural forms of ET, which are derived from pregnant mare's urine (Ruggiero and Likis 2002). CEE are the most widely prescribed HT for alleviation of postmenopausal symptoms and is considered to be a complex mixture, consisting of over 10 different estrogens (in their sulfate ester form), 200 metabolites and a number of other different compounds, including 7 progestins and 4 androgens (Notelovitz 2006). Some of the constituents of CEE have yet to be characterized, however the main components are: estrone sulfate (approx. 45%), equilin sulfate (approx. 25%), 17-α dihydroequilin sulfate (15%), delta8,9-estrone sulfate (3.5%), 17-β estradiol sulfate (1-2%), and 17-α estradiol sulfate (1-2%) to name a few (Notelovitz 2006).

Esterified estrogens are synthesized from CEE and, like CEEs, predominantly consist of E_1. These estrogens are dissolved in oil and are administered intramuscularly (Ansbacher 2001). Ethinyl estradiol is highly potent and is the most widely used compound in the oral contraceptive pill (de Lignieres and Silberstein 2000). It is synthesized from plant steroids and is biochemically similar to E_2, however it contains an ethinyl radical on carbon 17, which inhibits 17-β hydroxylation, thus having a strong antigonadotropic effect (de Lignieres and Silberstein 2000). For postmenopausal women who have not had a hysterectomy, a progestogen is often combined with ET for the purpose of protecting the endometrium from hyperplasia. The most common is medroxy-progesterone acetate, which has been speculated to negate the positive cardiovascular effects of ET (de Lignieres and Silberstein 2000). Selective Estrogen Receptor Modulators (SERMs) are a different newer class of ET. SERMs are synthetic non-steroidal drugs designed to target only specific types of tissue, where they bind to estrogen receptors and act as agonists or antagonists (Ruggiero and Likis 2002). Currently there are only two types of SERMs on the market; tamoxifen which is used in the treatment of breast cancer, and raloxifene which is used for treating osteoporosis (Ruggiero and Likis 2002).

Two important factors to consider when comparing the effects of one type of ET to another are: the route of administration and the dosage, which are interrelated. All oral estrogen preparations undergo first-pass hepatic metabolism in the liver and gut, resulting in a significant decrease in bioavailable E_2, resulting in a higher level of E_1 compared to E_2 (see Table 2 for serum E_1 and E_2 levels with different ET preparations of varying doses). It is estimated that an average of only 5% of orally administered E_2 (such as E_2 valerate and micronized E_2) is systemically bioavailable, thus rendering it more alike to the CEE and esterified estrogens than one would have initially presumed. However, research shows that 65% of the E_2 and 54% of the E_1 that enters circulation are converted to estrone sulfate, some of which (approx. 14% and 21%) can later be converted back to E_1 and E_2 respectively (Notelovitz 2006). Oral ET also stimulates production of SHBG, causing lower concentrations of free E_2 (Gleason et al 2005). Transdermal E_2 has very different pharmacokinetics to oral ET, given that it bypasses hepatic metabolism and is absorbed into the bloodstream at a steady-state (Gleason et al 2005). Therefore the bioavailability of E_2 is greater,

resulting in an E_1-E_2 ratio that is similar to the premenopausal state. Furthermore, a much smaller dosage of transdermal E_2 is required to raise circulating E_2 levels to that seen with oral ET preparations (e.g. 100 µg/day transdermal E_2 is approx. equivalent to 1.25 mg CEE) (Chetkowski et al 1986) (see Table 2). When interpreting serum E_2 levels after ET it should be kept in mind that the degree to which estrogens are synthesized locally in non-reproductive organs/tissue (e.g. adipose tissue, bone etc.) varies greatly between individuals and varies from organ to organ within an individual (Notelovitz 2006). Thus, it is not surprising that there are large discrepancies in: natural endogenous plasma estrogen levels, the ways in which women respond to HT and the occurrence of menopausal symptoms from one woman to the next.

Table 2. Serum Estradiol and Estrone Concentrations Observed with Different Estrogen Treatment Preparations in Postmenopausal Women* *(adapted from Dören 2001, p35)*

Preparations	Daily dose (mg)	Estradiol (pmol/l)	Estrone (pmol/l)
Oral			
Micronized Estradiol	1	110-184	551-1101
	2	184-180	1101-3120
Estradiol Valerate	1	184	587
	2	220-257	679-1101
CEE	0.625	110-184	551
	1.25	147-220	440-734
Parenteral			
Estradiol patches	0.05	110-239	147-165
	0.1	184-330	110-239
Gel	1.5	147-367	330
	3.0	220-514	165-569
Vaginal			
Micronized estradiol	0.5	918	477
CEE	1.25	25-40	239-294

CEE – conjugated equine estrogens, * Approximate concentrations reported using conversion factor: pmol/l divided by 3.67 = pg/ml (see Appendix A for equivalent pg/ml values).

1.3.3 Genomic Actions of Estrogens

Jensen and colleagues (1962) were the first to discover the intracellular estrogen receptor α (ERα) in 1962. Today there are two recognized types of ERs, α and β, the later of which was only recently discovered in 1996 (Kuiper et al 1996; Mosselman et al 1996). However, it is suspected that there are undiscovered membrane-related isoforms, given that some effects of estrogen cannot be attributed to either ERα or ERβ (Singh et al 1999). Estradiol has the highest binding affinity for both ERα and ERβ (see Figure 3 for binding potency of E_1, E_2 and E_3 for ERα and ERβ). It has been suggested that ERβ may be more likely linked to cognitive effects than ERα, given that in ERβ knock-out (KO) mice, sexual differentiation, fertility and lactation are not hindered in any way by the absence of this receptor subtype (Krege et al 1998). Furthermore, the ERβ isoform is more prominent than ERα within the cerebral cortex (Deitch et al 2001; Kritzer 2002). The examination of the distribution and possibly differential roles of these two receptor subtypes is beyond the scope of this review (for further discussion see McEwen 1999; Pettersson and Gustafsson 2001; Treuter et al 2000).

Intracellular (also termed 'classical') ERs have been extensively researched and identified in numerous cell-types throughout the body. They have been located not only in reproductive organs and the hypothalamus-pituitary but also in cells of the kidneys, lungs, prostate, muscle, bone, midbrain, brain stem, amygdala, pre-optic area, septum, hippocampus and cerebral cortex (for a review see McEwen and Alves 1999). A third classical ER known as ER-γ (an isoform of ERβ), has recently been discovered in the fish brain and ovaries, but has not been found in mammals (Hawkins et al 2005; Hawkins et al 2000; Sabo-Attwood et al 2004). ERs are members of the 'Nuclear Receptor' super-family, which are a large group of enhancer proteins that share a highly conserved structure and commonly influence gene transcription. As a result, estrogens can have either a direct effect on gene transcription or an indirect effect via second messenger systems.

The direct genomic mechanism requires estrogen (free or albumin-bound) to cross the cell membrane bi-layer via passive diffusion. Once in the cytoplasm estrogen

enters the nucleus (it is unclear whether estrogen moves freely or requires a transporter protein to enter the cell nucleus) and binds to the nuclear form of either ER subtype, creating a ligand-receptor transcription factor (Lee and McEwen 2001). Nuclear ERs contain 6 domains (named A-F). The three major functional domains are: (1) the N-terminus (comprised of domains A and B) which is responsible for cell-specific gene transcription via it's Activation-Function-1 (AF-1), (2) the central deoxyribonucleic acid (DNA)-binding domain (DBD; comprised of the C domain) which interacts directly with the DNA helix, and (3) the C-terminal ligand-binding domain (LBD, comprised of domain E) which contains the transcriptional Activation-Function-2 (AF-2) (Klinge 2000; Kraus and Wong 2002). It should be noted that while ERα and ERβ share all the same functional domains and structure (with ERβ having slightly fewer amino acids [530] than ERα [595] and lacking a portion of the C-terminal domain), their degree of homology is low considering they are subtypes of a common receptor. In particular the homology of the LBD is approximately 55% (Enmark and Gustafsson 1999). This implies that different synthetic estrogens may in the future be designed to specifically target one of the two ER subtypes and not the other. Once the ER is ligand-bound it becomes activated by inducing conformational change in the receptor protein, thus triggering dimerization. ERα and ERβ can form either homodimers or heterodimers. The ligand-receptor complex then binds to estrogen response elements (ERE) in the tissue-specific promoter region on the long arm of chromosome 6 for ERα, or chromosome 14, band q22-24 for the ERβ isoform (Enmark and Gustafsson 1999). The binding of the ER to it's specific ERE is thought to be dependant on phosphorylation and may often require the stimulation of nuclear factor kappa-B (NF-κB), activation protein-1 (AP-1) response elements and AP-1 transcription factors, namely c-fos and c-jun (Klinge 2000). The initiation of transcription after the ligand is bound to its receptor is complex and involves the recruitment of various cofactors at the site of the promoter region of the target gene. There are three general classes of cofactors, these being: (1) co-activators (proteins which interact directly with ERs to enhance transcription), (2) co-repressors (proteins which repress gene activation by maintaining chromatin (primarily DNA) in a condensed state, and (3) chromatin remodelers (Klinge 2000; Kraus and Wong 2002). ERs only bind to co-repressor when they are not ligand-bound. Once the initiation has been completed, ribonucleic acid (RNA) polymerase

II is recruited and transcription takes place. In addition, it has been found that estrogen, once bound to ERα, can also influence gene transcription by interaction with other DNA-bound transcription factors (Klinge 2000). This direct genomic process requires at least 45 minutes for the synthesis of new proteins and 1-2 hours to alter cellular response (Barnea and Gorski 1970).

Indirect genomic actions of estrogens are less well-defined and thought to involve the stimulation of a second messenger system, possibly via plasma membrane-associated ERs. While cell surface estrogen binding sites were first discovered in 1977 (Pietras and Szego 1977), the chemical structure of such a receptor has yet to be defined. Thus, its existence continues to be debated. Despite this, there is overwhelming evidence to suggest estrogen binds to membrane receptors, which will be discussed later in this chapter. It has been speculated that membrane ERs are most likely coupled with G-proteins and that the G-protein-coupled receptor-dependent mechanism then triggers a second messenger (Kelly and Wagner 1999; Thomas et al 2006). Second messenger systems that estrogen has been found to activate include: protein kinase A, B and C (PKA, PKB or Akt and PKC respectively), mitogen-activated protein kinase (MAPK) plus the MAPK isoform extra-cellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3K) as well as adenylyl cyclase (AC), and cyclic guanosine monophosphate (cGMP) (Ahmad et al 1999; Ansonoff and Etgen 1998; Aronica et al 1994; Brown et al 2001; Carrer et al 2005; Frigo et al 2006; Kato et al 1995; Kelly et al 1999; Lagrange et al 1997; Mannella and Brinton 2006; Palmon et al 1998; Razandi et al 1999; Russell et al 2000; Thomas and Dong 2006; Vasudevan et al 2005; Watters et al 1997; Watters et al 2000). Activation of any of these second messengers can lead to phosphorylation of numerous proteins and transcriptional regulators. For example, when estrogen activates the PKA pathway it triggers the phosphorylation of cyclic adenosine monophosphate (cAMP) response-element binding-protein (CREB), which can then regulate gene transcription by interacting with the regulatory region cAMP response element (CRE) on a DNA sequence. Similarly, by estrogen activating the MAPK or ERK second messenger it triggers the phosphorylation of the serum response factor (SRF)-Elk-1 complex, which then binds to its respective response element and alters gene transcription (Lee and McEwen 2001).

Subsequently, interaction with response elements such as these can also lead to cross-talk and interaction with other genes that are non-responsive to estrogen (i.e. lack the ERE) (see Figure 5 for depiction of putative mechanisms of estrogen's actions).

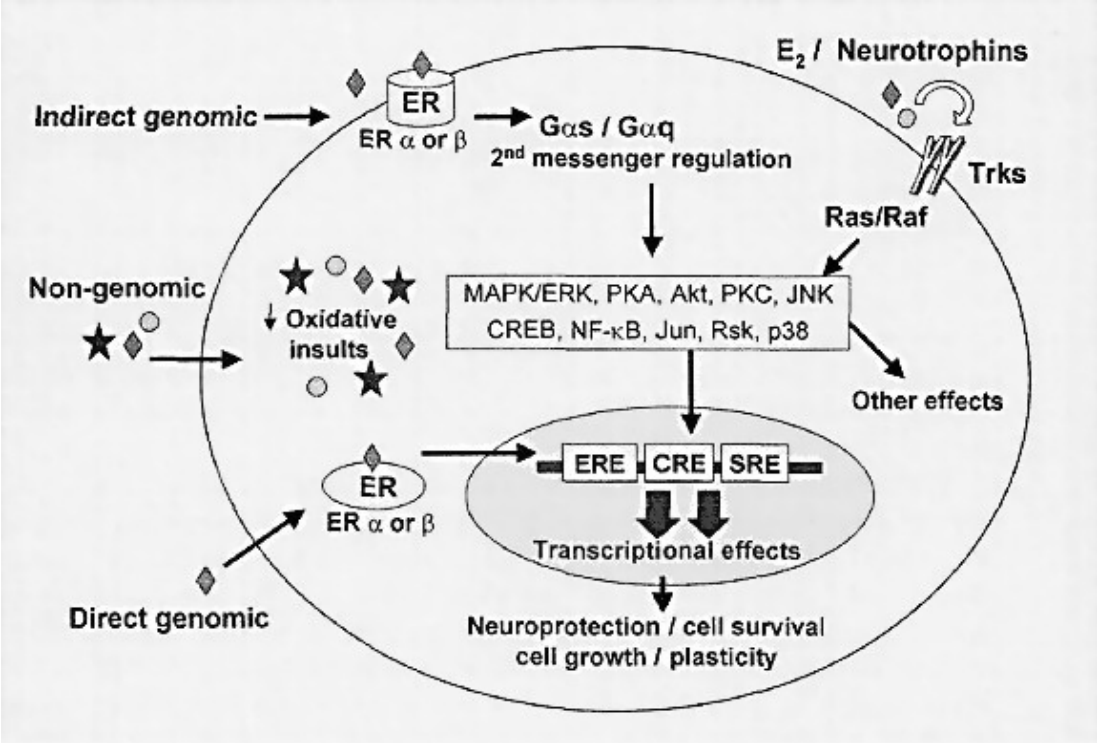

Figure 5. Putative Mechanisms of Estrogen Actions *(adopted from Lee and McEwen 2001, p573).*

Given estrogen's neurotrophic-like effects, it has been speculated that estrogen may also initiate the MAPK cascade using the same mechanisms as growth factors, via activation of the transmembrane *trk* receptors (Toran-Allerand 2004). This pathway, once activated involves the binding of the *trks* to the signaling enzyme Ras, usually via interaction with intracellular proteins such as PKC, c-Src, SHC and Grb2/SOS. Activation of Ras leads to the activation of the enzymes/kinases in the following order: Rap-1, Raf, MEK1/2 and ERK. Extra-cellular signal-regulated kinase then translocates to the cell nucleus where it can interact with various nuclear substrates to indirectly regulate gene transcription. Alternatively, ERK may activate cytoplasmic

proteins, such as Rsk, which can then enter the nucleus and act as a mediator for altering gene transcription (Toran-Allerand 2004).

The majority of early research into the indirect genomic actions of estrogen has been on oocytes, pituitary and hypothalamic cells, neuroblastoma cells and carcinoma cells. These cell lines are related to reproductive functions, motivated behavior, responses to stress or development of cancer. In addition, a growing body of literature has also found estrogenic activation of second messenger cascades in midbrain (Beyer and Karolczak 2000; Ivanova et al 2002), basal forebrain (Dominguez et al 2004) and cortical neurons (Honda et al 2000; Mannella and Brinton 2006; Setalo et al 2002; Singer et al 1999; Singh et al 2000; Toran-Allerand et al 2002). Estrogen's ability to activate such cascades (eg. the MAPK pathway) provides evidence in support of the neuroprotective hypothesis given that these pathways play an important role in survival, differentiation and proliferation of neurons in the CNS (Maggi et al 2004). Moreover, estrogenic activation of these cascades and the subsequent neuroprotective effects in cortical and hippocampal neurons has great implications for the preservation and possible enhancement of cognitive processes such as learning and memory. However, research in this area is in its infancy and currently speculative in regards to the role of putative membrane ERs. Therefore, future research is this area is sure to flourish and will hopefully see the cloning and identification of the membrane ER 'ER-X', as termed by Torran-Allerand and colleagues (2002).

1.3.4 Non-Genomic Actions of Estrogens

Incidentally, some non-genomic effects of estrogens may also involve the activation of the second messenger systems and signal transduction cascades described above. However, rather than leading to transcription they lead to non-genomic effects, such as changes in metabolic functions and morphology of cytoplasmic structures. Much attention has fallen upon non-genomic mechanisms which support the existence of cell-surface membrane ERs, where rapid cellular activation by estrogen occurs within seconds to minutes (Toran-Allerand et al 2002). Specifically, research using whole-cell patch-clamp techniques has shown physiological levels of E_2 to inhibit

calcium (Ca^{2+}) currents in neostriatal and sensory neurons of rats (Lee et al 2002; Mermelstein et al 1996). This effect was not only more pronounced in female compared to male neurons but was also steroid-specific, where E_2 was more effective than other types of estrogens (Lee et al 2002; Mermelstein et al 1996). Conversely others have found E_2 to induce a rapid Ca^{2+} influx in hippocampal neurons and that this influx was required for E_2 to initiate the Src/ERK signaling cascade, activation of CREB and the subsequent increase in Bcl-2 protein expression (Wu et al 2005; Zhao et al 2005). The Bcl-2 protein is an important antiapoptotic protein involved in regulating mitochondrial-mediated cell survival. These findings support E_2's neuroprotective hypothesis as Ca^{2+} has been shown to mediate regulation of neurite outgrowth and synaptic plasticity (Mattson 1990; Redmond et al 2002; Wakade et al 1995). Others have found E_2 to rapidly increase sodium (Na^{+})-mediated currents and attenuate outward potassium (K^{+}) currents in rat hypothalamic neurons, thus increasing neuronal excitability (Kow et al 2006). Similarly, E_2 has been found to modulate K^{+} channel activity in rat cortical neurons (Zhang et al 2005a), and hypothalamic neurons of the guinea pig (Kelly et al 2002).

Estradiol has also been found to increase the amplitude of excitatory postsynaptic potentials (EPSPs) as well as enhance long-term potentiation (LTP; sustained neuronal firing lasting up to several days) in rat hippocampal slice cultures (Foy et al 1999; Kim et al 2002; Kim et al 2006; Teyler et al 1980). Whole-cell recording techniques have shown significant increases in the amplitude of kainate-induced currents in CA1 hippocampal neurons after E_2 treatment (Gu et al 1999; Moss and Gu 1999). Furthermore, this increase was similar for both wild-type and ERα knock out (ERαKO) mice. In addition, potentiation was unaffected after the ER antagonist ICI 182,780 was employed to block ERβ and ERα, providing further support for the existence of another, likely membrane-related, ER isoform (Gu et al 1999). In addition E_2 has also been shown to induce hippocampal LTP *in vivo* (Cordoba Montoya and Carrer 1997; Good et al 1999). LTP in the hippocampus is thought to play an important role in the acquisition of information and long-term memory storage (for review see Lynch 2004).

The ability of acute E_2 to alter neuronal excitability in the hippocampus and cortex has implications for cognitive function, not only in relation to the protection of these brain regions but by possibly enhancing the efficiency of encoding and storage of information into long-term memory. Furthermore, Ca^{2+} plays an important role in the release of various neurotransmitters, such as dopamine and acetylcholine, which govern specific higher-order cognitive processes. Calcium can also activate certain signaling cascades which can then lead to changes in intracellular structures. Research into genomic and non-genomic mechanisms of estrogen's actions in the brain are on-going, but have thus far provided strong support for estrogen as a neuromodulatory agent (for more extensive review of these mechanisms see Boulware and Mermelstein 2005; Marin et al 2005; McEwen 2001; Sak and Everaus 2004; Toran-Allerand 2004). In parallel, further research into the underlying mechanisms of estrogens has focused on proliferative effects and the neuroprotective hypothesis.

1.4 Estrogen and Neurogenesis

Plasticity of the human brain and modulation of synapse and dendrite formation by estrogen had previously been thought to occur predominantly during the early developmental stages of life. However, evidence now shows hormone-regulated plasticity continues throughout adulthood (McEwen and Woolley 1994). Of particular relevance are findings of increased dendritic spine and synapse density in CA1 (a subfield of the cornu ammonis/dentate gyrus) pyramidal cells within the hippocampus during the proestrous (high estrogen) stage of the rat estrus cycle (Woolley et al 1990), and following E_2 treatment (Gould et al 1990; McEwen and Woolley 1994). More recent evidence found neurite outgrowth in the hippocampus required E_2 levels higher than that provided by the gonads, adding further evidence of local estrogen synthesis in the brain (Fester et al 2006; von Schassen et al 2006). The ability of estrogen to induce new spine and synapse formation in the brain is well documented (for reviews see Cooke and Woolley 2005; Murphy and Andrews 2000), however the mechanisms underlying these effects have yet to be defined.

There are a number of possible mechanisms by which estrogen may induce the formation of new excitatory spine synapses. One proposed method is via activation of the class of inotrophic glutamate receptors, the N-methyl-D-aspartate (NMDA) receptors. This theory was based on the evidence that NMDA receptor antagonists blocked estrogen-induced synaptogensis (Woolley and McEwen 1994), and NMDA receptor density increased in the CA1 region of the hippocampus following E_2 treatment (Weiland 1992; Woolley et al 1997). Estradiol has also been found to increase immunoreactivity of a subunit protein of the NMDA receptor, NR1, in the cell bodies and dendtrites of hippocampal neurons (Gazzaley et al 1996). Estradiol's ability to increase NMDA receptor sensitivity and synaptogenesis has great implications for hippocampal-dependent memory formation and preservation, given that NMDA receptors are excitatory and have been implicated in regulation of Ca^{2+} influx and LTP (Skeberdis et al 2006). This, as mentioned earlier, is essential for learning and memory processes. Research into the effects of ET on NMDA receptor density in other brain regions is lacking. In contrast to the findings regarding the hippocampus, ovariectomy has been found to have no effect on NMDA receptor density in the frontal cortex and striatum, regions essential for higher-order cognitive functioning (Cyr et al 2000). Furthermore, the addition of ET actually caused a decline, rather than an increase, in NMDA receptor binding in these two brain regions (Cyr et al 2000). These differential findings suggest that estrogen's ability to induced NMDA receptor upregulation is region specific. Woolley and colleagues (2000) suggest that estrogen-related increases in NMDA receptor density and sensitivity may be dependent on synapse and spine plasticity, as hippocampal neurons contain plastic properties not found in neurons of other brain regions.

Estrogen's effects on neurogenesis may also be via interaction with growth factors and neurotrophins. Estrogen had been repeatedly shown to interact with, and share similar activation pathways as brain-derived-neurotrophic factor (BDNF) (for review see Scharfman and MacLusky 2006). Estrogen has been speculated to induce BDNF synthesis, given that the BDNF gene contains a functional estrogen-response-element (Sohrabji et al 1995), and ERα and BDNF have been found co-localized in hippocampal neurons (Solum and Handa 2002). Indeed, BDNF mRNA has been shown to decline post-ovariectomy and increase with ET in the hippocampus,

amygdala and cortex (Berchtold et al 2001; Gibbs 1998; Liu et al 2001; Singh et al 1995; Sohrabji et al 1995; Stoltzner et al 2001; Zhou et al 2005). Furthermore, BDNF mRNA and mRNA of the nerve growth factor (NGF) receptor trkA, have been found to fluctuate with the estrous cycle in the hippocampus and basal forebrain respectively (Gibbs 1998). Interestingly, high levels of BDNF and trkA mRNA did not correspond with the high estrogen (proestrus) phase of the rat estrous cycle. Instead, levels were higher during the low estrogen (diestrus) phase (which occurs 24 hours post estradiol surge), suggesting estrogen's actions on BDNF and TrkA are likely via a genomic mechanism and require at least 24 hours to exert its effects. TrkA mRNA levels have also been found up-regulated following ET (Gibbs 1998; Gibbs et al 1994; McMillan et al 1996). It is important to note that, in embryonic and young animals, BDNF mRNA levels do not always reflect BDNF protein levels which have actually been found to decrease with ET (Murphy et al 1998b; Solum and Handa 2002). Despite this, the estrogen-induced decrease in protein levels were found to correspond with an increase in dendritic spines (Murphy et al 1998b), suggesting an inverse relationship between BDNF protein and estrogen.

Estrogen's effects on the universal neurotrophin receptor p75 have been less consistent, as p75 expression has been found up-regulated in young adult and aged rats (Jezierski and Sohrabji 2001) as well as down-regulated in aged rats following ET (Ping et al 2002). However, the earlier of these two studies used a higher dosage of estradiol, approximately 17µg/day, as opposed to the 10µg/day used by Ping and colleagues (2002), suggesting a dose-dependent response. Furthermore, Jezierski and Sohrabji (2001) used aged 'ovariectomized' (OVX) rats whereas Ping and colleagues (2002) used non-cycling aged rats. Research suggests that aged female rats do not undergo ovarian failure as such, but do experience a change in gene expression (LeFevre and McClintock 1988). This is why overiectomy is often performed, to try and mimic the ovarian failure as well as the change in gene expression observed in postmenopausal women.

Another proposed mode of action for estrogen-BDNF-induced neurogenesis is via suppression of inhibitory γ-aminobutyric acid (GABA)-ergic interneunons. This has been suggested because ET has been found to decrease BDNF expression and

GABAergic activity (ie. glutamate decarbolaze; GAD) in inhibitory interneurons of hippocampal (Murphy et al 1998a) and cortical (Blurton-Jones and Tuszynski 2006) cultures. Inhibition of GABAergic interneurons by estrogen is said to lead to the disinhibition of adjacent pyramidal neurons, allowing continued neuronal activity of pyramidal cells, which is required for BDNF-induced dendritic spine formation in the hippocampus (Scharfman and MacLusky 2006). Further research into estrogen's effects on neurotrophins and their receptors is needed to determine the specific conditions under which neurogenesis is observed, and the length at which it can be sustained.

Insulin-like growth factor 1 (IGF-1), although not part of the classical neurotrophin family, is recognized as having significant neurotrophic effects (for review see Aberg et al 2006). Early research found insulin and ET synergistically increased neurite outgrowth in hypothalamic explant cultures of fetal rats (Toran-Allerand et al 1988). Not surprisingly estrogen and IGF-1 have been found to share similar activation pathways, such as the MAPK cascade pathway. Their receptors have not only been found co-localised in various cell types of the brain but ERs are capable of being activated by IGF-1 and visa versa (for review see Cardona-Gomez et al 2001; Mendez et al 2005; Mendez et al 2006). These findings lead researchers to propose that estrogen and IGF-1 are interdependent and that their presence, or perhaps the co-activation of their receptors, is required for synaptic plasticity and neuronal differentiation to occur. While the majority of research in this area has focused on estrogen and IGF-1 in synaptic remodeling and neuritic outgrowth during early development and in adulthood, few studies have investigated the neuroprotective effects of these two agents against injury and oxidative stress, which will be discussed later in this chapter.

Another protein that has been suggested to play an intermediary role in estrogen-related neurogenesis is the growth associated protein GAP-43, a membrane bound protein involved in aiding axonal outgrowth and motility during synapse formation (for review see Benowitz and Routtenberg 1997). Specifically, GAP-43 mRNA has been found co-localized with GRH in neurons of the hypothalamus and fluctuates with the rat estrous cycle, with the highest expression occurring on the day of

proestrus (Prevot et al 2000). Research has also shown GAP-43 mRNA expression to decrease after ovariectomy and to increase with ET in the preoptic area, hypothalamus, but not the cortex (Lustig et al 1991; Shughrue and Dorsa 1993; Singer et al 1996a). Similar effects of ET have been seen in the basal forebrain of male rats (Ferrini et al 2002). Other researchers studying male and female postnatal rats suggested testosterone together with activation of ERs and androgen receptors may modulate GAP-43 expression (Shughrue and Dorsa 1994). In particular, their finding of increased GAP-43 mRNA expression in the cortex of females after dihydrotestosterone (DHT; a metabolite of testosterone which is unable to be converted to estrogen), together with the finding of decreased expression in males following tamoxifen (antiestrogen), suggests that steroid modulation of GAP-43 is gender specific. Overall, very little is known about the interaction of the GAP-43 protein with estrogenic mechanisms and any influence they may have on neurogenesis is at this stage largely speculative.

Estrogens' modes of action in cell neurogenesis are not well defined. Its ability to promote proliferation may be influenced by the dosage of estrogen, with an acute moderate dose (10µg) but not a low (1µg) or high (50µg) dose inducing rapid proliferation of cells in the dentate gyrus of ovariectomized rats (Tanapat et al 2005). In addition, a time-dependent affect has been found where the acute administration of E_2 was only effective when given 1 week after ovariectomy as opposed to 4 weeks post-ovariectomy. These two factors should be considered when assessing protective or enhancing effects of estrogen on behavior and cognitive performance, which will be discussed further in Chapter 2.

1.5 Neuroprotective Mechanisms of Estrogens

Neuroprotective effects of estrogen have implications for neuronal repair, age-related cognitive decline and neurodegenerative disorders such as AD and Parkinson's Diseases. In addition, estrogen's neuroprotective effects have also been implicated in other neurological disorders, such as schizophrenia. One of the most remarkable actions of estrogens is the suppression of oxidative stress from various neurotoxins. Neurotoxins, such as hydrogen peroxide (H_2O_2), induce lipid peroxidation causing a

build-up of free radicals within the neuron eventually causing cell death (Lee and McEwen 2001). Specifically, *in vitro* studies have found pre-treatment with E_2 for 2-24 hours protected against oxidative stress induced by the neurotoxins H_2O_2, buthionine sulfoximine (BSO), β- Amyloid (Aβ), ferrous chloride ($FeCl_2$) and ferrous sulfate ($FeSO_4$) in cultured cortical and hippocampal neurons of rodents (Bae et al 2000; Behl et al 1997; Goodman et al 1996; Keller et al 1997; Numakawa et al 2006; Singer et al 1996b). Similarly, oxidative damage related to excessive levels of glutamate (3-10μm), has also been found suppressed in cortical and hippocampal slice cultures, and H22 cells, pretreated with E_2 (Behl et al 1997; Goodman et al 1996; Harms et al 2001; Mize et al 2003; Nilsen and Brinton 2002; Yi et al 2005).

17-β Estradiol is not the only estrogen capable of protection from oxidative stress as E_1, E_3 and a number of estrogen derivatives, such as ethinyl estradiol, have also protected cortical (Bae et al 2000; Keller et al 1997) and hippocampal neurons from these neurotoxins (Behl et al 1997). However, it appears that for any estrogen to have an antioxidant effect the phenolic structure must remain intact (Behl et al 1997; Manthey and Behl 2006). In addition the enantiomer 17-α estradiol, an inactive estrogen thought to be incapable of ER binding, has also been found to protect mouse hippocampal neurons and HT22 cells from oxidative stress induced by Aβ (Behl et al 1997). This suggests that the protective effects of estrogens may not be mediated via the classical intracellular ERs. This is supported by later research showing pretreatment with 17-α estradiol not only protected hippocampal neurons from oxidative stress but cortical cultures as well (Bae et al 2000; Mize et al 2003). In line with the non-nuclear ER-mediated theory for neuroprotection is the finding that HT22 cells transfected with a mutated form of ERα (incapable of mediating ERE transcription) were protected against glutamate toxicity with E_2 treatment (Mize et al 2003). Furthermore within 15 minutes E_2 rapidly phosphorylated ERK2 in these cells, further supporting the existence of membrane-related ERs and their importance in neuroprotection.

A number of possible mechanisms for estrogens' antioxidant effects have been put forward, including the induction of lipid peroxidation and estrogens acting as scavengers for free radicals. However, the evidence for initiation of various cascades

such as CREB and MAPK has been most outstanding (Manthey and Behl 2006). Specifically, Fitzpatrick and colleagues (2002) found E_2-mediated neuroprotection from oxidative stress was dependent on phosphorylation of ERK2, given that the MEK inhibitor PD98059, blocked this effect. This finding is further supported by the findings of Mize and colleagues (2003), mentioned above. Similar results were observed with protein phosphatase inhibitors in rat cortical neurons (Yi et al 2005). A later study by Numakawa and colleagues (2006) specifically found E_2 protected cortical neurons against H_2O_2-induced oxidative stress by reducing MAPK activity (which becomes overactive with H_2O_2, leading to cell death), and the subsequent accumulation of intracellular Ca^{2+}. Further research into the biochemistry behind estrogens' antioxidant effects is required. Worth noting is that some suggest neuroprotection only occurs when the estrogen (regardless of type) is administered prior to induction of oxidative stress (Behl et al 1997). However others have found a protective effect even when the estrogen (including estrone, 17α- and 17β-estradiol) was administered concurrently with the neurotoxin (Bae et al 2000; Yi et al 2005). Both high (10-30 μM) and low (10-100 nM) concentrations of E_2 have been found to elicit neuroprotection (Bae et al 2000; Behl et al 1997).

In addition to a protective effect from oxidative stress, estrogen has also been found to significantly attenuate the extent of necrotic and apoptotic cell death, induced by methods such as exposure to NMDA, artery occlusion or oxygen-glucose deprivation, in numerous brain regions including the cerebral cortex (Dubal et al 1999; Rau et al 2003; Toung et al 1998) and hippocampus (Bagetta et al 2004; Cimarosti et al 2005; Raval et al 2006). This area of research has been extensively reviewed with strong consistent evidence for a protective effect of estrogen against ischemia, thus it will not be reviewed here (for review see McCullough and Hurn 2003; Merchenthaler et al 2003; Yang et al 2003). However, the proposed neuroptoective mechanisms that may govern these effects are not well-defined. Estrogen's interaction with IGF-1 is one suggested mode of action. Specifically, research has found E_2's neuroprotective effects to be blocked with the IGF-1 receptor antagonist JB-1, in lesioned dopaminergic nigrostriatal neurons of OVX rats (Quesada and Micevych 2004). These findings support those of Azcoitia and colleagues (1999b) who also found the protective effect of E_2 to be abolished when

administered with JB1 or the ER antagonist ICI 182,780, after kainic acid induced hippocampal lesions. In addition, they also found that the neuroprotective effect of IGF-1 alone was also blocked by ICI 182,780, suggesting estrogen and IGF-1 may act synergistically to protect and attenuate the extent of damage incurred after insult or trauma.

A proposed mechanism that has been more extensively investigated involves Apolipoprotein E (ApoE), a glycoprotein that has long been associated with neuronal protection and membrane repair (for review see Teter 2004). *In vitro* and *in vivo* studies have found estrogen treatment to: (1) increase neuronal sprouting after entorhinal cortex lesions in wild-type mice but not ApoE-KO mice (Stone et al 1998), (2) attenuate ischemic damage in the caudate nucleus for non ApoE-deficient mice only (Horsburgh et al 2002), (3) increase expression of ApoE mRNA in mouse brain (Srivastava et al 1996) and (4) up-regulate ApoE in the cortex, diencephalon and hippocampus of ovariectomized mice (Levin-Allerhand et al 2001) and in cultured cortical neurons (Nathan et al 2004). In addition, Struble and colleagues (2003) found ApoE to fluctuate with the mouse estrous cycle, with the lowest levels observed during estrus (low estrogen phase). However, this was specific only to the hippocampus, cingulate and frontal corticies as the opposite relationship was found in the olfactory bulb and cerebellum, implying ET effects on ApoE are region-dependant.

The exact mechanism by which estrogen interacts with ApoE to promote neuroprotection is not known. Research suggests ApoE up-regulation in the cortex specifically, may be via interaction with ERβ or a non-classical ER (Levin-Allerhand et al 2001). Similarly, evidence suggests ApoE up-regulation by ET in the olfactory bulb and cerebellum may also be via the ERβ isoform (Struble et al 2003). Despite this, down-regulation of ApoE and ApoE mRNA have been found using the ERβ-selective agonist, diarylpropionitrile, in HT-22 transfected cells (Wang et al 2006). Conversely, up-regulation of these markers was seen with the ERα agonist propylpyrazole (Wang et al 2006). It may be that estrogen's effects on ApoE vary depending on the cell type being targeted. Research also suggests ET effects on ApoE are transient (McAsey et al 2006). McAsey and colleagues (2006) finding up-

regulation of ApoE expression in the whole brain 5 days after continuous E_2, but not 14 days after treatment. This is in line with the majority of research in this area which gave continuous E_2 (of varying concentrations) for 5 days and found similar results (Levin-Allerhand et al 2001; Srivastava et al 1996; Srivastava et al 1997; Struble et al 2003). Lastly, one of the genetic risk factors for developing AD (and various other neurological disorders) is the ApoE4 isoform, produced by the epsilon 4 allele (Mahley et al 2006). Nathan and colleagues (2004) found estradiol increased neurite outgrowth of cultured cortical neurons for transgenic mice modified to express ApoE3, but there was no effect of estradiol on the cultures taken from ApoE4 transgenic mice. This suggests ApoE genotype is something that should be considered when assessing the neuroprotective effects of HT and ET on dementia and neurological disease. Indeed, a prospective longitudinal study in aged women (>65 yrs) found HT significantly slowed the rate of cognitive decline in women who did not express epsilon 4, but had no effect for epsilon 4-positive women (Yaffe et al 2000).

Alzheimer's Disease is histopathologically characterized by the progressive build up of senile plaques which consist of Aβ peptides and neurofibrillary tangles. Given this, one focus of the literature has been to determine the underlying mechanism responsible for estrogen's protection against Aβ-induced cell death. The production of Aβ protein has been found reduced in animal and human cortical embryonic neuron cultures following E_2 treatment (Xu et al 1998). In addition, the cleaved soluble fragment of the Aβ precursor protein (sβAPPα; cleaved by the enzyme α-secretase which inhibits the formation of $A\beta^{1-40}$ and $A\beta^{1-42}$ peptides, the main constituents of neurofibrillary plaques), has been shown to increase after ET, further suggesting a neuroprotective effect against Aβ production (Xu et al 1998; Xu et al 2006; Zhang et al 2005b). Xu and colleagues (2006) also found that in transgenic mouse models of AD, mice that were deprived of estrogen showed a marked increase in brain $A\beta^{1-42}$ than non-estrogen-deprived mice, and that this increase was reversed by 50% after E_2 treatment. These findings are consistent with previous research (Levin-Allerhand et al 2002; Yue et al 2005; Zheng et al 2002). Other lines of evidence have suggested estrogen acts by: (1) suppression of Aβ precursor protein signaling to the nucleus and thus inhibition of transcriptional regulation and

subsequent apoptosis (Bao et al 2007), (2) preventing proteolytic cleavage of tau (a prominent protein found in neurofibrillary tangles) (Park et al 2007), (3) inhibition of Aβ-induced down-regulation of Bcl-2 expression (Nilsen et al 2006), and (4) inhibition of Bax (a protein involved in signaling apoptotic activities) translocating from the cytosol to mitochondria (Nilsen et al 2006). Worth noting is that estrogen dosage may influence neuroprotection against Aβ, as an *in vitro* study found high dose (200ng/ml) E_2 had no protective effect and actually exacerbated Aβ-induced cell death, which was the opposite effect of low dose (10ng/ml) E_2 (Chen et al 2006). Low dose E_2 also protected against neurodegeneration regardless of whether it was administered acutely, continuously or intermittently. This suggests a temporal pattern of administration is not a factor in neuroprotection against Aβ toxicity. Lastly, 8 weeks of transdermal E_2 (0.1mg/day) was found to significantly reduce $A\beta^{40}$ plasma levels compared to baseline for postmenopausal women with AD who were HT-naïve (Baker et al 2003), further supporting a direct effect of estrogen on Aβ production.

Needless to say these findings have many implications for preservation of cognitive function in older age and the protection against AD and related dementias. Research in this area has now begun to investigate the role of estrogen's interaction with glia cells. This is because glia cells have important roles in regulatory mechanisms in the brain, such as glucose metabolism (for review see Escartin et al 2006). They have also been implicated in neuroplasticity and neuroprotection (for reviews see Allen and Barres 2005; Mahesh et al 2006; Markiewicz and Lukomska 2006). Findings that support a neuroprotective effect of estrogen via glia cell modulation include; (1) localisation of ERs in microglia and astrocytes (the most abundant type of glial cells in the brain) in various brain regions of animals and humans (Azcoitia et al 1999a; Langub and Watson 1992; Melcangi et al 1999; Milner et al 2001; Savaskan et al 2001), (2) reduction of microglia pro-inflammatory activities with ET (Bruce-Keller et al 2000; Dodel et al 1999; Liu et al 2005) and (3) increases in the expression and release of neuroprotective cytokines from cortical astrocytes following ET (Dhandapani et al 2005; Sortino et al 2004). Research also suggests astrocytes may play a role in neuroprotection by synthesis of estrogens locally as a response to brain injury. This is given that astrocytes in various brain regions, including the cortex and

hippocampus, have been found to express immunoreactivity for aromatase in animals with kinaic acid-induced lesions but not intact controls (Garcia-Segura et al 1999). In line with an injury-specific response is the finding of up-regulation of ERα expression in astrocytes following brain injury in monkeys (Blurton-Jones and Tuszynski 2001). Collectively, these findings suggest estrogens may modulate glia function in a way that ultimately reduces brain inflammation, which is often a factor in neurologic and neurodegenerative disorders (Godbout and Johnson 2006). (For more information on the anti-inflammatory hypothesis of estrogen neuroprotection see Dhandapani and Brann 2002; Mahesh et al 2006).

1.6 Summary

The molecular mechanisms of estrogens are complex and diverse. Not only are estrogens classical transcription factors but they also have non-genomic mechanisms for altering cell functioning, with strong evidence to support the existence of a rapid member-related ER isoform. While research in this area is in it's infancy, E_2 has been shown to exert novel effects on cortical and hippocampal neurons, including activation of various kinases and signaling cascades, modulation of Ca^{2+} and K^+ currents, and potentiation of neuronal excitability. Discovered in recent years was estrogen's ability to promote neurogenesis in the adult brain, the underlying mechanisms of which have been suggested to involve modulation of and interaction with NMDA receptors, BDNF, trkA, p75, GABAergic activity, IGF-I, and GAP-43. However, some of the most profound actions of estrogens have been those directly relating to the neuroprotective hypothesis and include: (1) protection from oxidative stress induced by H_2O_2, BSO, Aβ, $FeCl_2$, $FeSO_4$ and glutamate, (2) protection from necrotic and apoptotic cell death caused by NMDA exposure, artery occlusion and oxygen-glucose deprivation, and (3) the reduction in Aβ protein synthesis. Again, a number of mechanisms by which estrogen exerts these effect have been proposed, most interesting are those which engage IGF-I, ApoE and glia cells.

Worth consideration is the limitation of *in vitro* experimentation. Given that the properties and functioning of cultured neurons and astrocytes may differ from those in their natural state, the chemical reactions to estrogens can only be inferred to occur

in the same way *in vivo*. Notably however, this methodology gains insight into the possible cellular mechanisms of estrogen's actions that may not have been otherwise detectable.

In addition, although the majority of research reviewed in this chapter focused on the rapid effects of acute ET, some research suggests estrogen's modulation of certain neurochemicals may be transient (McAsey et al 2006), may be specific to certain brain regions (Struble et al 2003) and may depend on the cell type being examined (Levin-Allerhand et al 2001; Struble et al 2003; Wang et al 2006). While estrogen has emerged as a multifaceted neuromodulatory agent with various lines of research to support the neuroprotective hypothesis, much research is needed before the underlying mechanisms of estrogens' actions can truly be defined. Despite this lack of definitive conclusions, there is the unyielding consensus within the neurosciences that estrogens have positive neuroprotective effects on the brain and CNS, the implications of which are widespread.

SECTION TWO:
ESTROGEN, COGNITION & THE ROLE OF THE CHOLINERGIC SYSTEM

Chapter 2

The Effects of Estrogen on Cognition in Animals and Humans

2.1 Estrogen and Cognition: An Introduction

It is now well-established that estrogen has undoubtedly numerous positive biological effects on neuronal survival and brain functioning, as evidenced in the previous chapter. The cognitive effects of estrogens are however less clear-cut, and whether estrogen exerts a positive, negative or no effect on cognition continues to be a topic of great debate. The cognitive effects of estrogen treatment (ET) first fell under the spotlight during the late 70s and has achieved growing interest due to the proposition that estrogen may delay the natural occurrence of cognitive deterioration accompanying old age (Sherwin 2006). This has many implications for the use of estrogens before, during and after the menopause given that today's average life expectancy for Australian women is 82.8 yrs (UNSD 2007[Note]), suggesting approximately one-third of a woman's life is in an estrogen-deficient state.

The term 'cognition' is quintessentially used when referring to global mental function and includes all mental processes related to attention, acquisition, storage, transformation, retrieval and application of knowledge. While not all cognitive processes are affected by aging, there are a number that do decline significantly with age including fluid reasoning, mental speed, memory and spatial abilities (Keller 2006; Sherwin 2006). It has been suggested that some processes are more severely impaired with age than others, however there is little consensus over which cognitive process is more affected as working memory, memory recall, executive function, mental speed and verbal memory have all been proposed (Keller 2006; Sherwin 2006). Overall, the cognitive processes that deteriorate with age are essential used in everyday functioning, whether it be remembering a doctor's appointment, driving a car or communicating with others. Therefore many of the explorations of estrogen's effects on cognition have focused on the above cognitive domains. In particular estrogen's effects on declarative (also known as explicit) memory, namely recall and recognition which require the deliberate retrieval of an explicit prior event or stimuli from stored memory, has been the area of primary interest. The focus on memory has predominantly been due to age-related changes but also due to the clinical implications surrounding Alzheimer's Disease (AD) and related dementias. More recently the effects of ET on such cognitive process have also become relevant to the

Note: UNSD (2007): United Nations Statistical Devision Common Database: Life expectancy by sex: United Nations, New York.

field of mental illness, in particular schizophrenia research, as treatment for cognitive deficits are currently lacking. This chapter will now provide a summary and critique of previous literature addressing estrogen's effects on cognitive behavior in both animals and humans.

2.2 The Effects of Estrogen on Cognition in Animals

2.2.1 Sex Differences

Spatial abilities have been one of the most widely studied areas of gender research in both animals and humans. As to be expected it is also one of the most reliable domains in which gender differences are observed, where males consistently outperform females on spatial tasks (for reviews see Jones et al 2003; Williams and Meck 1991). Early research in rodents found males to perform significantly better than females on various spatial maze tasks, such as the Lashley III and symmetrical closed-field mazes (Williams and Meck 1991). However the majority of mazes used in early studies were ill-equipped to control for sex differences in locomotor activity, as females tend to engage in more exploratory behaviors than males, thus rendering them more likely to incur errors in these tasks (Archer 1975; Stewart et al 1975). Therefore, radial-arm and T-shaped mazes were introduced to assess spatial learning and memory, in an attempt to control for motor and exploratory behavior (given that the arms of these mazes are relatively narrow). Water maze tasks have also been extensively used to test spatial abilities as mice and rats instinctively try to escape to a platform as fast as possible. It has been repeatedly shown that males outperformed females in measures of spatial learning (Chen et al 2004; Frye 1995; Galea et al 1995; Galea et al 1994; Gresack and Frick 2003; Kavaliers et al 1998; Kavaliers et al 1996; Mishima et al 1986), spatial working memory (Gresack and Frick 2003) and spatial reference memory (Frick et al 2000; Gresack and Frick 2003; Seymoure et al 1996). Notably, some of these studies found sex differences only when females were expected to be in a high estrogen state (i.e. during proestrus phase or during breeding season) (Frye 1995; Galea et al 1995; Galea et al 1994), or if the animals were middle-aged (17 months) (Frick et al 2000). Despite this, a minority of researchers have found no sex differences in spatial learning (Bucci et al 1995) and spatial

working memory (Healy et al 1999) using the same types of mazes. Male superiority in spatial working memory performance has also been found using other types of tasks, such as a novel delayed object recognition task (Sutcliffe et al 2007). Interestingly, female rats performed the non-spatial version of the same task significantly better than the male rats (Sutcliffe et al 2007), which parallels previous research (Ghi et al 1999). Spatial divided attention has also been found to differ between the sexes, with male rats again performing significantly better than female rats (Jentsch and Taylor 2003). In line with rodent studies, young male rhesus monkeys have similarly shown better spatial working memory performance on a delayed recognition span test compared to females (Lacreuse et al 1999). Lacreuse and colleagues (1999) however, did not find any sex differences on any of the other tasks used, including an object memory task and measures of executive function. In summary, while there is strong evidence to support a gender bias in spatial learning and memory, little is known about sex differences in other cognitive functions, which requires further exploration.

2.2.2 Cycle Phase

Although investigation into the differences in cognitive performance between the sexes is an important area of research from an evolutionary perspective, it allows only inference into the specific hormones that may govern these processes. The natural fluctuation of estrogens with the estrous cycle provides a means for testing the hypothesis that estrogen may modulate certain cognitive functions. Consistent with gender research, estrous cycle phase has also been found to influence spatial memory and learning in rodents, with better performance during the estrus (low estrogen) phase (Warren and Juraska 1997). Spatial learning has been shown to be worse in female rodents with high compared to low estradiol levels (Galea et al 1995). Conversely, some researchers have observed poorer spatial learning during the estrus phase (low estrogen phase) (Frye 1995; Healy et al 1999), while others have found no differences in spatial learning or memory across the estrous cycle (Berry et al 1997; Stackman et al 1997). Lack of consistent results may be due to the more extensive training that animals in these studies received, given that pre-training has previously been found to eliminate the sex difference in spatial learning

performance (Perrot-Sinal et al 1996). Furthermore, although Stackman and colleagues (1997) concluded spatial working memory to be stable across the estrus cycle, they did find a significant increase in arm-choice latency during the proestrus (high estrogen) phase, which indicates a decrease in speed of information processing. Mixed findings may also be due to the use of different strains of rats [as hippocampal morphology has been found to fluctuate during the estrus cycle of Sprague-Dawley rats (Woolley et al 1990)], and/or the different versions of mazes employed across studies.

Extrapolating findings of estrus cycle variations in rodents to humans should be done with caution, given that endocrine and reproductive functioning differs considerably between species (eg. the rodent cycle spans a total of only 4 days). The rat estrous cycle consists of the proestrus phase (12 hours) followed by the estrus phase (36 hours), diestrus I (24 hours) and lastly diestrus II (24 hours). Estradiol levels begin to rise during diestrus I and peak during proestrus then return to baseline at the onset of estrus (Marcondes et al 2002). The transition of female rats to the 'estropause' is also very different to the human menopause as it is not characterized by a significant loss in estrogen production or by the failing of the ovaries. Instead, female rats experience irregular cycles which often result in either a state of persistent estrus (moderate levels of estrogens) or persistent diestrus/pseudopregnancy (high levels of progesterone) until anestrus or death (Huang and Meites 1975). Warren and Juraska (2000) found that aged female rats in constant estrus had better spatial learning performance than those in persistent diestrus, which is in line with their earlier study in young cycling female rats. However, there was no difference in estradiol level between the two groups, suggesting a different ovarian hormone may be involved. A more recent study using pregnant and virgin rats conversely found spatial working memory was better in pregnant animals which corresponded to significantly higher levels of estradiol and progesterone (Bodensteiner et al 2006), adding to the already incongruent literature. Research using non-human primates, namely Macaque monkeys which are most closely related to the human race, has found spatial recognition memory and visuospatial attention to be impaired during the pre-ovulatory (high estrogen) phase when compared to the luteal and follicular (lower estrogen) phases of the mensutrual cycle (Lacreuse et al 2001; Landauer et al 2004).

Further investigation into the relationship between menstrual cycle phase and spatial abilities as well as other cognitive processes in non-human primates is needed (see Lacreuse 2006).

2.2.3 Ovariectomy

Given the variability in estrogen levels across the estrus cycle, and the limited capacity to infer findings from estrus cycle research in rodents to the female (human) menstrual cycle, an alternative experimental strategy was employed. It investigated the effects of ovariectomy as a means of mimicking the human menopause. Research investigating the effects of ovariectomy, and thus the loss of endogenous estrogens, has produced mixed results. One research group initially failed to demonstrate a decline in learning and memory post-ovariectomy and instead found improved performance on one aspect of the object discrimination task in monkeys (Voytko 2000). In a later study however, they found a significant drop in visuospatial attention 2 months after surgery (Voytko 2002), which contradicts menstrual cycle and gender research, where lower estrogen levels correspond to better spatial performance. Markowska and Savonenko (2002) found that the longer the duration of estrogen withdrawal (1 vs 7 months) the greater the decline in performance on a delayed nonmatching-to-position (DNMP) task when compared to sham-operated (ovary-intact) control rats (although working memory was not assessed prior to ovariectomy). Similar detrimental effects of ovariectomy have been observed on spatial working and reference memory (Heikkinen et al 2002) and learning (Singh et al 1994) in rodents. Research in aged ovariectomized (OVX) monkeys has also found visuospatial working memory performance, as well as object recognition, to be worse when compared to healthy young monkeys (Rapp et al 2003a). This however, is not unexpected considering age-related cognitive decline. Similar studies comparing aged-OVX monkeys to intact age-matched pre-menopausal controls have also shown significantly poorer performance on delayed working memory tasks in OVX animals (Lacreuse et al 2000; Roberts et al 1997). In addition some researchers have failed to find any difference between OVX and intact rodents on measures of object recognition (Vaucher et al 2002) and spatial divided attention (Jentsch and Taylor 2003).

These findings suggest a negative effect of ovariectomy on various cognitive processes, including those which involve spatial abilities. This is incongruous to the theory generated from gender research where lower estrogen levels are thought to facilitate better performance on spatial tasks. However, in assimilation with menstrual cycle research, findings in this area are quite mixed. The main objective for ovariectomy of animals in this field of research has been for the purpose of controlling circulating estrogen levels and allowing the manipulation of hormonal status by administering exogenous hormone treatment, similar to that given to postmenopausal women in the form of ET/EPT. Thus, more research investigating the effects of estrogen on cognition has utilized a between-groups design using OVX animals treated with estrogen.

2.2.4 Behavioural Effects of Estrogen Treatment in Rodents

Of the 37 experimental studies that have aimed to examine the cognitive effects of ET in OVX animals, 78% found significant positive results, while 14% found an impairing effect (4 of these studies found both positive and negative results) and 11% failed to find any effect of ET on performance (for summary of cognitive behavioral research involving OVX female animals treated with estrogen see Appendix B). This body of literature is extremely diverse in terms of their experimental design, as researchers used varying doses, modes of administration, durations of treatment, and cognitive tasks.

2.2.4.1 Task Type and Cognitive Domain Assessed

Fry and Rhodes (2002) conducted one of the largest of the rodent studies using Long-Evans rats. They found E_2 enhanced performance on an inhibitory avoidance task, a measure of spatial learning and memory. In addition, neither tamoxifen nor ICI 182,780 (ER antagonists) administered in conjunction with E_2, were able to suppress performance enhancement, implying that the effects of E_2 on cognition may not be via classical nuclear ERs. This is consistent with biochemical evidence supporting the existence of membrane-bound estrogen receptors (Singh et al 1999; Toran-Allerand et al 2002), as discussed in Chapter 1. Similarly, a number of other studies that also used avoidance tasks have found learning to be significantly

enhanced in estrogen treated animals compared to controls (Das et al 2002; Frye and Rhodes 2002; Singh et al 1994). These findings support the reported positive effects of ET on learning and memory performance in water maze tasks (Bimonte-Nelson et al 2006; Daniel et al 2005; Frick et al 2002; O'Neal et al 1996; Packard and Teather 1997). As mentioned earlier, these tasks are associated with some level of stress, which has been suggested to be influenced by hormone levels, given female rats have been found to show improved cognitive performance after chronic stress (Bowman et al 2001). Conversely, the performance of male rats has been impaired following stress (Luine et al 1994). Sex-specific alterations in the levels of certain neurotransmitters and metabolites in the frontal cortex, amygdala and hippocampus have been proposed to modulate stress-related effects on cognition. This may possibly be dependent on the organizational and activational effects of E_2, although it has yet to be determined (for review see Luine 2002). Bowman and colleagues (2002) examined the role of estrogen in the female rats' response to stress. It was found that E_2 treated OVX animals that underwent daily restraint stress for 21 days performed better than all other groups (including the non-stressed E_2 group) on a spatial memory task (Bowman et al 2002). Therefore, it may be possible that inhibitory avoidance performance improved after E_2 partially due to the influences on stress. Despite these findings, numerous studies have found significant positive effects of ET (predominantly E_2) on non-aversive tasks such as the T-maze (Fader et al 1998; Heikkinen et al 2002), radial arm maze (RAM) (Fader et al 1999; Heikkinen et al 2002), delayed matching-to-position (DMP) and DNMP (Gibbs 1999; Gibbs 2000b; Markowska and Savonenko 2002) and object recognition tasks (Vaucher et al 2002), measures of working memory, reference memory and learning respectively. Overall, findings suggest that ET alone can enhance performance without the additional influence of stress.

As mentioned earlier a small handful of studies have found a negative effect of ET on cognition. One possible cause for this may lie with the type of cognitive strategy used to complete the task, as certain tasks appear to be differentially affected by estrogen treatment. Korol & Kolo (2002) found learning to be enhanced by ET during a 'place' task yet performance was impaired for the 'response' task. Both tasks were set in a plus-shaped maze with 4 arms, one containing a food reward. In

the 'place' task the reward remained in the fixed location while the rat was positioned in a different arm for each trial, thus leading them to discover the food by using extra-maze room cues (thus presumably using allocentric strategies). The 'response' task involved alternating the food reward and the animals' start location so that rats had to make either a left or right turn to reach the goal arm (thus presumably using egocentric strategies). The same results were found by Davis and colleagues (2005) using an 8-arm RAM task. The findings from these studies suggest estrogen's effect on learning depend on the cognitive strategies required to perform the task, and that estrogen has a positive effect on spatial reference learning and memory. Other studies that tested place-related learning skills, using tasks such as the Morris water maze and RAM, have consistently detected significant improvements in performance after ET (Bowman et al 2002; Fader et al 1999; Heikkinen et al 2002; Packard and Teather 1997). These studies provide further evidence for task-dependent effects of estrogen treatment with an advantage for tasks requiring allocentric strategies, implying enhanced spatial learning/memory and working memory. In addition, given the biochemical evidence of estrogen receptor mechanisms in the hippocampus (see Chapter 1), and that place learning tasks have been strongly linked to the hippocampus (for review see Silva et al 1998), it can be implied that estrogen modulates hippocampal functioning. This is supported by findings of enhanced cognitive performance with ET when directly injected/infused or implanted into the hippocampus (Frye and Rhodes 2002; Sinopoli et al 2006; Zurkovsky et al 2007). Estrogen-induced enhancement of cognitive performance may be dependent on the brain regions it acts upon. Improvements in working memory and learning have been found when ET was localized to the PFC or hippocampus (Frye and Rhodes 2002; Sinopoli et al 2006), but when infused into the striatum ET has been shown to impair learning (Zurkovsky et al 2007). This is again likely to have been due to the strategy used to complete the task (a response learning task in a Y-shaped RAM) as performance was only impaired when extra-maze cues were removed (see Appendix B for specific details regarding variations in the types of estrogens preparations administered).

2.2.4.2 Does Estrogen Treatment have a Dose- or Time-Dependent Effect?

Some researchers have suggested that ET has a dose-related effect on cognition. Packard and Teather (1997) found a dose-dependant effect of ET (Estradiol-cyclodextrin) on memory with 0.2 mg/kg (an intermediate dose) being optimal. The low (0.1 mg/kg) and the high (0.4 mg/kg) doses however, also resulted in significantly better performance compared to controls. Estrogen's effects were reportedly time-dependant, with memory enhancement occurring only after immediate post-training injection and not when estrogen was given two hours post-training. This suggests that the results were not due to estrogen influencing non-mnemonic factors, or swim speed. Similarly, Frick and colleagues (2002) found aged female mice to improve on a spatial learning and memory task with a 5µg dose of E_2 but not a 1µg dose. A later study by Fry and Rhodes (2002) showed significant improvements in rats that were administered a high dose (1mg) of E_2 compared to controls. This improvement was similar and no better than the group which received the 10µg dose, suggesting a non-linear relationship between dose and performance, where performance may plateau once a certain level of estrogen is achieved.

Dose-dependent effects have also been put forth as a possible cause for negative effects of ET, as high dose E_2 Benzoate (EB) (Foster et al 2003; Galea et al 2001; Korol and Kolo 2002) as well as low-dose EB (Sinopoli et al 2006) has been shown to significantly impair performance on measures of retention and working memory. This implies that an optimal level (possibly an inverted U-shaped response) of estrogen in the brain is required for positive effects on cognition. Interestingly, Sinopoli and colleagues (2006) found a dose-by-region interaction where high dose E_2 infused directly into the PFC facilitated working memory while the same dose infused into the hippocampus resulted in impaired performance. Furthermore, low dose E_2 infused into the hippocampus was more effective in facilitating working memory than either the high dose or placebo (Sinopoli et al 2006). Despite these findings, the literature predominantly displays significant enhancing effects with high, low and intermediate doses of ET, with no definitive evidence for a dose dependent-effect at present (see Appendix B for further information).

Treatment duration has also been postulated as a determining factor for significant ET effects (Gibbs 1999). Singh and colleagues (1994) found that as little as 2 weeks of ET was sufficient to enhance active avoidance relative to OVX controls, but that longer duration (25 weeks of ET) was more effective as performance was superior to that of gonadally-intact controls and OVX animals**.** Heikkinen and colleagues (2002) similarly found female OVX mice to show improved performance on the RAM and T-maze tasks with either 7 or 40 days of ET, with greater enhancement evident after chronic treatment (i.e. 40 days). Interestingly, sham-operated female mice as well as gonadally-intact male mice demonstrated enhanced learning on the RAM task after ET. This suggests that estrogen can have a positive effect even when there has not been a decline in/or disruption to endogenous estrogen levels, which gives rise to the possibility that estrogen may be useful as a treatment of cognitive deficits and not just as a replacement therapy. Given that various doses and durations (ranging from 3 days -10 months; see Appendix B) of ET have been found to enhance performance in rodents, it was suggested that discrepancies in the literature may not be due to duration of treatment but rather length of time between ovariectomy and initiation of treatment (Gibbs 2000b). Indeed, Gibbs (2000b) found ET initiated either immediately or 3 months post-ovariectomy enhanced acquisition of a DMP task but not when treatment was initiated 10 months post-ovariectomy. Authors argued that ET initiated soon after ovariectomy protected from cognitive decline associated with loss of endogenous estrogens. This limited window of opportunity may be related to neuronal cell loss and integrity of the cholinergic system following ovariectomy, which will be discussed in Chapter 3.

2.2.5 Behavioural Effects of Estrogen Treatment in Non-Human Primates

The majority of animal research has involved female rodents, however more recent research has been conducted on OVX monkeys. Voytko (2000) investigated the effects of 5 and 16 months of ET on measures of learning and memory in macaca fasicularis monkeys, yet found no significant effects. This may have been due to the finding that 2 months post-ovariectomy (just prior to treatment) performance had not changed from the pre-operative level. Additionally, the OVX monkeys that received placebo unexpectedly failed to show a drop in performance 12 and 24 months post-

ovariectomy. Voytko (2002) in a later study found that, for estrogen treated monkeys, reaction time (RT) on a visuospatial cuing task (measure of attention) was significantly faster after 4 months of treatment. However, these findings are limited as there were no differences in accuracy measures between estrogen and placebo groups, possibly due to the task being too simple, as both groups demonstrated approximately 97% accuracy post-treatment. In contrast Rapp and colleagues (2003a) found visuospatial memory and recognition memory to be restored following cyclic E_2 treatment in comparison to vehicle-treated OVX monkeys. Furthermore, these improvements were not likely to be a result of secondary effects on motivational or perceptual abilities, as no differences were found on an object discrimination task designed to test these factors. Lacreuse and colleagues (2002) also found memory to be enhanced following ethinyl estradiol. This was only evident with the spatial delayed recognition span test and not for the delayed response or DNM-to-sample tasks, implying that ET effects may be specific to spatial memory or memory processes predominantly reliant on the hippocampus. Performance was significantly enhanced after 2-4 months but not after 6-8 months, which may be due to practice effects or a decline in motivational and novelty aspects of the task (which was administered repeatedly for 9 months). The lack of significant treatment effects on the other tasks may be due to the sample size being too small (N=5) to detect discrete differences between treatment conditions. Furthermore, the fact that animals had been OVX for 10-16 years and had not received any hormone treatment in the last 3 years leading up to the study (and one had never received any ET) may have also contributed to the lack of significant results. Neuronal deterioration associated with ovariectomy (see Chapter 3) may have been too extensive for ET to restore functioning of certain brain regions. A later study revealed a detrimental effect of estrogen on a face recognition task, while no effect was found on the spatial or object versions of the same task (Lacreuse and Herndon 2003). It was suggested that estrogen heightened emotional responsiveness to face recognition because the stimuli was of social relevance (Lacreuse and Herndon 2003). Studies of this nature are limited but thus far support a beneficial effect of ET on visuospatial memory and recognition. Further research encompassing a broader range of cognitive domains, including learning and working

memory, is needed to better understand the effects of ET on cognition in non-human primates.

2.2.6 Summary of Animal Research

Although comparability between behavioral ET studies (see Appendix B) is difficult due to the variability in doses, treatment regimen (acute versus chronic), type of estrogen and form of administration used, it can be concluded that ET does appear to have positive effects on cognition. In particular, spatial learning and memory and working memory appear to benefit the most, given that the majority of researchers found significant results, despite the differences in methodology. The effect of estrogen on 'motivation' has been suggested to be a confounding factor, given that many tasks involve motivational cues such as a food reward as in most RAM and T maze tasks. However motivation is unlikely to have such an impact on results given that ET has also been shown to have positive effects on the water maze tasks (which don't use motivational cues). In summary, there is a plethora of behavioral animal research suggesting estrogen has a positive effect on cognitive processes, in particular learning, reference memory and working memory. Currently there are no definitive conclusions regarding dose- or duration-specific effects. An optimal level of estrogen in the brain however, has been proposed, with emerging evidence suggesting a possible dose-by-region interaction where the optimal level of estrogen within the hippocampus may differ to that of the PFC**.** Further research is needed to determine this possibility. Collectively, the animal literature has established a strong foundation for estrogen as a possible modulator of cognitive functions. In parallel, the behavioral literature investigating estrogen treatment in humans is also vast, yet less consistent.

2.3 The Effects of Estrogen on Cognition in Humans

2.3.1 Gender Differences

Sexually-dimorphic organization of the brain, in terms of development and laterality (for reviews see Voyer 1996; Wisniewski 1998), lead to the conception of divergent

cognitive functioning for men and women. While not all cognitive processes are gender-specific, there is a long line of literature supporting women as generally having superior performance on tasks of verbal abilities, perceptual speed and accuracy, and fine motor skills, while men perform better on spatial and mathematical tasks and in tasks requiring gross motor strength (Caplan et al 1985; Collaer and Hines 1995; Linn and Petersen 1985; Owen and Lynn 1993; Roberts and Bell 2000). Unlike animal research, gender differences in human cognition have been extensively investigated with the above summation being the general consensus within the field, thus it will not be reviewed here (for comprehensive reviews see Halpern 1992; Hyde et al 1990; Voyer et al 1995). Research investigating naturally occurring hormonal fluctuations over the course of the menstrual cycle with relation to cognitive functioning has however been more variable.

2.3.2 The Menstrual Cycle and Cognitive Performance

Based on findings from gender research, it was hypothesized that phases of the menstrual cycle characterized by higher estrogen levels would result in better performance on cognitive tasks that favor females and poorer performance on tasks that typically favor males. A number of studies support this theory with findings of poorer performance on spatial tasks, deductive reasoning and mathematical tasks during the mid-luteal or late follicular (high estrogen) phases of the cycle as compared with the time of menstruation when estrogen is low (Hampson 1990a; Hampson 1990b; Hampson and Kimura 1988; Hausmann et al 2000; Maki et al 2002; Phillips and Silverman 1997). In support of the above theory are findings of better performance on measures of articulation, verbal fluency, manual speed/coordination, perceptual speed/attention and working memory during high estrogen phases of the cycle (Broverman et al 1981; Hampson 1990a; Hampson 1990b; Hampson and Kimura 1988; Maki et al 2002; Rosenberg and Park 2002; Symonds et al 2004; Ward et al 1978). Conversely some findings contradict the gender hypothesis with the opposite effect occurring on measures of verbal processing (Ussher and Wilding 1991), verbal fluency (Symonds et al 2004), spatial encoding (Postma et al 1999) and implicit memory (Maki et al 2002). Moreover, the majority of researchers, including many of those just mentioned, have failed to find

significant menstrual cycle-related differences on numerous cognitive measures. These include memory and typical gender-specific tasks, involving such processes as mental rotations, mental arithmetic, verbal recall, verbal fluency and spatial skills (Fernandez et al 2003; Gordon and Lee 1993; Kasamatsu et al 2002; Maki et al 2002; O'Reilly et al 2004; Phillips and Sherwin 1992b; Rosenberg and Park 2002; Symonds et al 2004; Ussher and Wilding 1991).

Discrepancies between studies are possibly due to variations in methodology. Invariably the most common issue in research of this kind has been the way in which menstrual cycle status was determined as some used self-report measures (Hampson 1990b; Phillips and Silverman 1997; Postma et al 1999; Symonds et al 2004), others used basal body temperature (Broverman et al 1981; Kasamatsu et al 2002; Rosenberg and Park 2002), while the majority used hormone assays (Gordon and Lee 1993; Hampson 1990a; Hausmann et al 2000; Maki et al 2002; O'Reilly et al 2004; Phillips and Sherwin 1992b; Ward et al 1978), the latter being the most reliable. Furthermore, one study did not exclude women who were taking oral contraceptives (Postma et al 1999), bringing findings from this study into question. Worth noting is recent evidence suggesting that circulating plasma/serum E_2 levels do not accurately reflect E_2 levels in the brain, given that certain brain regions produce their own estrogens locally (Simpson 2003). In support of this are the grossly inconsistent findings from correlation research. Significant positive correlations of plasma or serum E_2 levels have been observed with measures of verbal fluency, verbal memory, and executive functioning (Drake et al 2000; Farrag et al 2002; Maki et al 2002; Wolf and Kirschbaum 2002), while significant negative correlations have been found between estradiol levels and scores on mental rotation and visual memory tasks (Drake et al 2000; Hausmann et al 2000; Maki et al 2002). Although these findings support menstrual cycle research the majority of researchers have failed to find any significant relationship between E_2 levels and cognitive performance (Almeida et al 2005; Gordon and Lee 1993; Halari et al 2005; Herlitz et al 2007; Janowsky et al 2000; O'Reilly et al 2004; Phillips and Sherwin 1992b; Polo-Kantola et al 1998; Portin et al 1999; Yaffe et al 1998; Yonker et al 2003). This implies that peripheral levels of circulating estrogens may not be analogous to estrogen levels in the brain. Conversely, Fernandez and colleagues (2003), using functional magnetic

resonance imaging (fMRI), found activation patterns of the medial superior frontal cortex during a semantic decision task to be significantly correlated with E_2 (and progesterone) serum levels. However, they found no variation in cognitive performance between different phases of the menstrual cycle, further questioning the reliability of behavioral menstrual cycle research.

There are a limited number of studies that have investigated the cognitive effects of contraceptive use in healthy cycling women. An early study by Wuttke and colleagues (1975) found that women taking a combined standard dose of EPT generally performed worse on reaction time and mathematical tasks, than normal cycling controls. The authors however, did not report between-groups statistics. Silber and colleagues (1987) in a later open-label cross-over study found no cognitive effects of a combined estrogen-progesterone contraceptive of varying doses. Gordon and Lee (1993) similarly found no differences in cognitive performance between healthy cycling women, women on combined oral contraceptives and women with amenorrhea (not due to gonadotropic abnormalities). In contrast to the lack of research in healthy young women, research investigating HT and cognitive function in postmenopausal women is abundant, yet incongruent due to a myriad of methodological limitations which will be discussed shortly. Despite inconsistencies in the literature, extensive analyses have led to the general deduction that ET has small positive effects on certain domains of cognitive function, namely verbal memory, abstract reasoning and information processing (for a meta-analysis see Hogervorst et al 2000; Zec and Trivedi 2002). This chapter will now briefly review and critique the literature involving HT and cognitive function in healthy postmenopausal women.

2.3.3 The Effects of Estrogen Therapy on Cognition in Healthy Postmenopausal Women: Findings from Observational Studies

In 2005 it was reported that, among Australian women aged 45 years and over, 11% (424,300 women) were at that time using HT prescribed by a doctor. Nearly two thirds (65%) of those women had been using HT for 5 years or more (ABS 2006[Note]). Research shows that age-related cognitive decline accelerates with the menopause

Note: ABS (2006): 2004-5 National Health Survey: Summary of results, Australia: Australian Bureau of Statistics.

(Halbreich et al 1995), leading to the suggestion that women receiving HT during and after the menopause are somewhat protected from this cognitive and neural deterioration. Barrett-Conner and Kritz-Silverstein (1993) conducted the first observational study in a community sample of postmenopausal women and found no differences in the rate of cognitive decline between past-users, current-users and never-users. Since then, a number of prospective/observational studies have found significant positive results. Overall, women taking HT have been found to perform better than non-users in the following cognitive domains; verbal fluency (Grodstein et al 2000; MacLennan et al 2006; Miller et al 2002; Szklo et al 1996; Yonker et al 2006), verbal and visual memory (Henderson et al 1996; Jacobs et al 1998; Kampen and Sherwin 1994; Keenan et al 2001; Maki et al 2001; Resnick et al 1998; Resnick et al 1997; Robinson et al 1994; Sherwin 1988; Smith et al 2001; Stephens et al 2006; Verghese et al 2000; Yonker et al 2006), working memory (Duff and Hampson 2000; Keenan et al 2001; Miller et al 2002) attention (Friebely et al 2001; Smith et al 2001), abstract reasoning (Jacobs et al 1998; Keenan et al 2001; Schmidt et al 1996), information processing (Kritz-Silverstein and Barrett-Connor 2002; Lokken and Ferraro 2006; MacLennan et al 2006) and global cognitive functioning (Kimura 1995; Matthews et al 1999; Rice et al 2000; Steffens et al 1999). There are also a number of studies that support the initial findings of Barrett-Conner and Kritz-Silverstein (1993), having found no significant benefit of HT-use on cognitive functioning (Alhola et al 2006; Galen Buckwalter et al 2004; Henderson et al 2003; Hogervorst et al 1999; Kang et al 2004; Kurt et al 2006; Low et al 2006; Morse and Rice 2005). A wide range of measures have been used to assess these cognitive domains, many of which are traditional paper and pencil tasks with good reliability and validity (see Table 3 for list of typical measures used to assess cognition).

A proportion of these observational studies not only report on differences between HT-users and non-users but also analyzed prospective data where follow-up assessments were compared to a previous assessment (at which point most HT-users had already begun treatment). Resnick and colleagues (1997) found visual memory to remain stable over approximately 2 years of ET compared to non-users who declined in performance. Significant improvements in performance on measures of verbal memory (Jacobs et al 1998), abstract reasoning, verbal fluency and global

cognition (Rice et al 2000), have also been seen after approximately 2.5 and 2 yrs of HT-use, respectively. Conversely, Alhola and colleagues (2006) found that continuous HT-use for 6 years resulted in decreased performance on a verbal skills task and on an immediate and delayed verbal recall task compared to non-users. However, little weight was given to these findings as only 4 of 45 variables were significant and no apparent corrections were applied for multiple comparisons (other than those pertaining to covariates).

In addition, a number of researchers have found no significant within-group effects of HT-use and cognition (with follow-up ranging from 4-6 years to approximately 14 years) (Barrett-Connor and Kritz-Silverstein 1993; Matthews et al 1999). More recently, Stephens and colleagues (2006) found that 3 months of HT resulted in improvements in global memory and verbal memory in young peri- and post-menopausal women. However, they did not use a control group. Although prospective analyses have yielded mixed results the majority of cross-sectional research did find positive effects of HT. Despite this, there are a number of confounding factors that need to be taken into account when drawing conclusions from this literature.

2.3.4 Methodological Limitations of Observational Studies

It is difficult to compare results from observational and epidemiological studies due to the large variation in study characteristics, in terms of sample size (eg. *N* range = 19 - 9000+), patient characteristics (i.e. clinical vs general population) and demographics (i.e. country of origin). Some studies involved younger postmenopausal women around the 40-60 age range (eg. Kimura 1995; Lokken and Ferraro 2006; Morse and Rice 2005), others comprised older post-menopausal women aged >75 who may have been without endogenous estrogens for 20+ years (eg. Galen Buckwalter et al 2004; Kang et al 2004; Robinson et al 1994), while some researchers specifically chose women with recent surgical menopause (ie. hysterectomy or oopherectomy) (Farrag et al 2002; Verghese et al 2000). Not only did participants vary in demographics between studies, but participants within studies were also quite diverse as some studies had large age ranges (eg. 42-67 yrs: Lokken

and Ferraro 2006; eg. 55-93 yrs: Robinson et al 1994) and used women who had been taking HT for varying durations (eg. range = < 6 mths- 20+ yrs: Maki et al 2001).

Table 3. List of Typical Cognitive Tasks used in Observational and Experimental HT Studies in Healthy Post-Menopausal Women

Cognitive Domain	Common Measures used in Previous Research
Verbal Fluency/Expression	Controlled Oral Word Association Test Category Fluency Test Boston Naming Test Vocabulary Test*
Verbal Learning & Memory	Bushke Selective Reminding Test California Verbal Learning Test Rey Auditory Verbal Learning Test Logical Memory/Paragraph Recall* Verbal Paired Associates*
Visual Memory	Figural Memory* Visual Reproduction* Visual Paired Associates* Benton Visual Retention Test
Working Memory	Digit Span*
Abstract Reasoning/Cognitive Flexibility	Similarities* Stroop Interference Arithmetic
Information Processing/ Psychomotor Speed	Trail Making Test (Halstead-Reitan Battery) Digit Symbol Substitution Test (DSST)* Simple RT (eg. CogniSpeed) Choice RT (eg. CogniSpeed)
Attention	Digit Span* Visual Memory Span* Visual Search Letter/Digit Cancellation Digit Symbol* Stroop Trail Making Test (Halstead-Reitan Battery) Simple RT (eg. CogniSpeed) Choice RT (eg. CogniSpeed) Vigilance test
Global Cognition	MMSE 3MSE

Note: * Subtests of the Wechsler scale/s ie. Wechsler Adult Intelligence Scale (WAIS), WAIS-Revised, Wechsler Memory Scale (WMS), WMS-Revised & WMS-III. MMSE-Mini Mental State Examination, 3MSE- Modified MMSE.

Notably, some studies examined the impact of treatment duration on cognition, which yielded mixed results. The majority of research has shown no relationship between duration of ET (range < 6 mths to >20 yrs) and cognitive performance in the domains of visual memory, verbal learning and memory, working memory, information processing, mental rotations and global cognitive functioning (Henderson et al 2003; Kimura 1995; Kurt et al 2006; Low et al 2006; MacLennan et al 2006; Maki et al 2001; Miller et al 2002; Resnick et al 1997; Steffens et al 1999). Conversely, Szklo and colleagues (1996) found word fluency to improve slightly with longer duration of treatment (9-44 yrs) in surgically menopausal women but not naturally menopausal women. Grodstein and colleagues (2000) also found a strong association between duration of HT-use and verbal fluency scores for women who had been using for ≥ 5 years but not in women who had been using for ≥10 years. Matthews and colleagues (1999) found better digit symbol performance in women taking HT for ≥ 10 years compared to women who had been on treatment for < 10 years, but no significant effects were observed on the 3MSE and Trails B measures.

These inconsistent findings may be due to a number of reasons. Firstly, initiation of treatment after the menopause has recently been suggested as a critical factor which may determine whether or not ET has a protective effect on cognitive processes (for review see Craig et al 2005; Markou et al 2005; Sherwin 2007). Therefore, it may be that negative findings are due to women initiating treatment too late after the loss of endogenous estrogens and possibly after cognitive deterioration has begun to take place. Secondly, some researchers failed to differentiate between ET-users and EPT-users, as the addition of progesterone to an ET regimen has been suggested to have detrimental effects on cognition. Indeed, mental tracking, and global cognitive functioning have been found impaired with EPT, relative to estrogen-only treatment (Rice et al 2000). The opposite has been found on the card rotations test, a measure of spatial ability (Maki et al 2001). The possible disparate effects of adjuvant progesterone treatment is still under debate as the majority of findings have shown no difference between ET and EPT on cognitive functioning (Galen Buckwalter et al 2004; Grodstein et al 2000; Kampen and Sherwin 1994; Low et al 2006; MacLennan et al 2006; Miller et al 2002). Other factors that may explain confounding results relate to the 'healthy user bias' as women who elect to use HT during and after the

menopause tend to be healthier than non-users, of a higher socioeconomic status and have undergone more years of education (for a review see Hogervorst et al 2000; Zec and Trivedi 2002). Hormone treatment effects on mood, subjective well-being and climacteric symptoms have also been suggested to inadvertently influence cognitive performance however reviews of the literature have found no consistent evidence of secondary effects on cognition (for a review see Hogervorst et al 2000; Zec and Trivedi 2002).

Despite the methodological variation between studies, it should be noted that the majority of researchers took the following confounding factors into consideration by either matching samples or controlling for them statistically; age and education (Alhola et al 2006; Barrett-Connor and Kritz-Silverstein 1993; Farrag et al 2002; Galen Buckwalter et al 2004; Grodstein et al 2000; Henderson et al 2003; Hogervorst et al 1999; Kampen and Sherwin 1994; Kang et al 2004; Keenan et al 2001; Kimura 1995; Kurt et al 2006; Lokken and Ferraro 2006; Low et al 2006; MacLennan et al 2006; Maki et al 2001; Miller et al 2002; Morse and Rice 2005; Rice et al 2000; Robinson et al 1994; Smith et al 2001; Steffens et al 1999; Szklo et al 1996; Verghese et al 2000; Yonker et al 2006), time since menopause (Grodstein et al 2000; Keenan et al 2001; Smith et al 2001; Szklo et al 1996), socioeconomic status (Kampen and Sherwin 1994; Kang et al 2004; Maki et al 2001; Miller et al 2002; Szklo et al 1996; Verghese et al 2000) and ethnicity (Galen Buckwalter et al 2004; Szklo et al 1996; Verghese et al 2000). Overall, findings support the estrogen protection hypothesis as significant effects have been observed even after controlling for confounding factors, with the greatest support evidently for preservation of verbal memory.

Jacobs and colleagues (1998) for example, found that women who were currently taking or had ever taken HT performed significantly better on the Selective Reminding Test, Similarities (WAIS-R) and the Boston Naming Test when compared to women who had never used HT. When short-term (< 1year, M = 2.72 mths) and long-term (>1 year, M = 9.83 years) users were discretely compared to never-users, both short and long-term users still performed better on neuropsychological tests (after controlling for the above variables). Given the

possible acute effects of estrogen on cognition, Jacobs and colleagues (1998) excluded current users from a secondary analysis, which did not alter the results. This finding, plus the lack of a relationship between time of last estrogen use and time of cognitive testing (M = 24.5 years), suggests that HT may have a long-lasting positive effect on cognition even after treatment has ceased. This implies that structural or morpholological changes took place, which is in line with estrogen-stimulated proliferative effects and neurogensis in the brain (as outlined in Chapter 1). In further accordance with this, brain activation patterns during recognition memory tasks have been found altered after 2 years of HT, with an increase in regional cerebral blood flow observed in the hippocampus, parahippocampal gyrus, entorhinal cortex, insula, middle temporal and frontal lobes and the cerebellum compared to non-users (Maki and Resnick 2000).

Epidemiological and observational research has recently become more stringent in controlling for confounding variables that are difficult to assess such as mental health, stress, quality of life, exercise, alcohol use, surgical menopause, consistency of HT use (ie. continuous/intermittent), dosage and type of preparation (oral/transdermal), smoking, body mass index (BMI), blood pressure, diabetes, apoE genotype, use of vitamin E supplements, aspirin and non-steroidal medications (Grodstein et al 2000; Kang et al 2004; MacLennan et al 2006). However, randomized experimental trials continue to provide more reliable controlled environments to test the effects of estrogen treatment on cognitive processes.

2.3.5 The Effects of Estrogen Treatment in Healthy Postmenopausal Women: Findings from Controlled Trials

The first experimental trial, conducted by Caldwell and Watson (1952) and later followed-up by Caldwell (1954), found significant improvements on subscales of the Wechsler-Bellevue Intelligence Scale and WMS after 6, 12 and 18 months of ET. However, interpretation of these findings are limited as all women had a previous history of mental illness. In addition, although the treatment group routinely received a daily dose of 2mg estradiol benzoate (EB), after 6 months some women also received varying doses of a progestagen or testosterone. A later study by

Hackman and Galbraith (1976) also found 6 months ET (piperazine estrone sulfate) to improve performance on a measure of global memory. The age of the sample however, ranged from 29-68 years, and not all women were postmenopausal as 10 of the participants reportedly presented with 'estrogen deficiency'. Since then a number of well-controlled experimental studies have explored the effects of ET on cognition (see Table 4 for summary of experimental trials in healthy postmenopausal women). Eight of the 26 studies listed in Table 4 found a significant effect of ET on cognition, with two of these studies also finding a significant impairing effect on a different cognitive measure (Krug et al 2003; Wisniewski et al 2002). Improvements have been seen in the areas of verbal fluency (Shaywitz et al 2003), verbal learning/memory (Joffe et al 2006; Krug et al 2006; Krug et al 2003; Phillips and Sherwin 1992a; Sherwin 1988), visual learning/memory (Duka et al 2000; Linzmayer et al 2001; Wisniewski et al 2002), working memory (Krug et al 2006), abstract reasoning (Sherwin 1988), speed of information processing (Sherwin 1988), response inhibition/cognitive flexibility (Krug et al 2006) and spatial skills (Duka et al 2000). These studies used varying treatment durations (ranging from 3 days – 6 months), varying types of estrogen preparations (CEEs, esterified estrogens, E_2), different modes of administration (intramuscular (i.m)/oral/transdermal) and differing dosages (eg. 50µg vs 100µg/day transdermal E_2).

Some commonalities do exist between the studies that found positive ET effects. Firstly, these studies all administered estrogen without the addition of a progestagen which, as discussed previously, may be a significant determining factor of cognitive effects. Worth consideration is a study in healthy young women which found an acute dose of progesterone to have a detrimental effect on visual memory (van Wingen et al 2007). In line with these findings are results from animal and human studies where combined EPT, but no ET, were equivalent to a placebo or control group (Bimonte-Nelson et al 2006; Wegesin and Stern 2007). These results suggest that the addition of progestagen may mitigate the positive effects of ET.

Table 4. Experimental Studies that Examined the Effects of Estrogen Treatment on Cognition in Healthy Postmenopausal Women

Study	N	M age (range)	Design	Treatment	Mode	Duration	Measures	Outcome
Rauramo et al (1975)	88	46.90 (30-55)	Parallel, PC	2mg E_2	-	6 mths	Integrative memory test, SRT, CRT, Letter Cancellation (processing speed), Raven Progressive matricies (reasoning)	No effect.
Sherwin (1988)	50	45.40 (NR)	Cross-over, R, DB, PC	10mg/mth E_2 valerate OR 200mg/mth T OR 1mg EB + 7.5mg E_2 dienanthate + 150mg T/mth	i.m	3 mths	Digit Span, Paragraph Recall, Clerical speed (processing speed), Abstract Reasoning	All 3 HT regimens were associated with better cognitive performance on all tasks.
Ditkoff et al (1991)	36	53.00 (45-60)	R, DB, PC	0.625 OR 1.25mg CEE	-	3 mths	Digit Span, DSST	No effect.
Phillips & Sherwin (1992a)	19	48.20 (NR)	R, DB, PC	10mg/mth E_2 valerate	i.m	2 mths	Digit Span, Paragraph Recall, Associate Learning, Visual Reproduction	Verbal learning/ memory improved for the ET group compared to placebo.
Goebel et al (1995)	89	74.40 (>69)	R, DB, PC	0.625mg CEE + MPA	-	8 mths	TMT – Trails B (processing speed)	No effect.
Polo-Kantola et al (1998)	62	56.30 (47-65)	Cross-over, R DB, PC	2.5mg/day E_2 gel OR 50µg/day E_2 patch	Gel or patch	3 mths	Digit Span, DSST, BVRT, SRT & CRT tasks, Letter Cancellation (attention/ spatial skills), Stroop, Auditory Attention test	No effect.
Hogervorst et al (1999)	22	53.75 (>45)	Parallel	2mg/day E_2 + 2.5 OR 5mg P	-	12 mths	Word Recall (verbal memory & learning)	Memory improved in HT group but no difference relative to controls.
Shaywitz et al (1999)	46	50.80 (33-61)	Cross-over, R DB, PC	1.25mg/day CEE	Oral	21 days	Verbal and visual working memory tasks	No effect of ET on performance, but brain activation patterns were altered.

Table 4. Continued…….

Study	N	M age (range)	Design	Treatment	Mode	Duration	Measures	Outcome
Wolf et al (1999)	38	68.65 (NR)	DB, PC	100µg/day E_2	Patch	2 weeks	Verbal Fluency task, city map task (spatial memory), Paired Associates, Stroop, mental rotations	No effect of ET. For the ET group higher E_2 levels correlated with better verbal memory.
Duka et al (2000)	37	65.00 (55-75)	R, DB, PC	100µg/day E_2	Patch	3 weeks	Berlin Test of (visual) Associative Memory, Paired Associates Learning, Attentional Shift task, Stroop, Number Generation task, Tower of London, Mental Rotation Task	Memory & spatial abilities improved for the ET group, relative to the placebo group.
Janowsky et al (2000)	13	69.05 (61-74)	R, DB, PC	0.625mg/day CEE	Oral	1 mth	Subject Ordering Pointing Test (working memory)	No effect.
Binder et al (2001)	52	81.50 (75-91)	R, DB, PC	0.625mg/day CEE (+ 5mg/day MPA for women with non-surgical menopausal)	Oral	9 mths	Paired Associate Learning, Animal Category (word fluency), TMT, Letter & Figure Cancellation tests (attention/spatial ability)	No effect.
Wisniewski et al (2002)	26	58.00 (46-77)	R, DB	1.25mg/day esterified estrogen alone OR + 2.50mg methyl-T	Oral	4 mths	Identical Pictures (recognition memory), Cube Comparisons, Building Memory, Shape Memory (spatial learning & memory)	Visual recognition improved for the ET group but worsened for the ET +T group. Spatial learning/ memory worsened for the ET group only.
Rapp et al (2003b)	4381	NR (>65)	R, DB, PC	0.625mg/day CEE + 2.5mg/day MPA	Oral	*M* = 4.2 yrs	3MSE	Global cognition worsened for the EPT group compared to the placebo group.

Table 4. Continued…….

Study	N	M age (range)	Design	Treatment	Mode	Duration	Measures	Outcome
Shaywitz et al (2003)	60	51.20 (32-64)	R, DB, PC	1.25mg/day CEE	Oral	21 days	Gray Oral Reading Tests, Paired Associate Learning, Sentence Span, Boston Naming Test, COWAT, figural memory task, mental rotations, Letter Cancellation test	Oral reading was better in the ET group relative to the placebo group.
Krug et al (2003)	24	58.05 (47-65)	Cross-over, DB, PC	100µg/day E_2 OR 6mg/day T	Patch	3 days	Divergent and convergent thinking tests, immediate verbal recall	ET impaired divergent thinking but enhanced convergent thinking & immediate memory compared to placebo.
Espeland et al (2004)	2808	NR (65-79)	R, DB, PC	0.625mg/day CEE	Oral	*M* = 5.4 yrs	3MSE	The ET group declined in performance on the 3MSE compared to placebo.
Joffe et al (2006)†	52	51.05 (40-60)	R, DB, PC	50µg/day E_2	Patch	3 mths	CVLT, WMS-R, Rey-Osterreith Complex Figure, spatial & verbal working memory tasks	The ET group had fewer preservative errors on the CVLT after treatment relative to placebo.
Smith et al (2006)	10	56.90 (50-60)	Cross-over, R, DB, PC	5µg/day ethinyl E_2 + 1mg norethindrone acetate	Oral	4 weeks	Visual Delayed Matching to Sample Task (spatial working memory)	No ET effect on performance but PFC activation increased with ET.
Krug et al (2006)	14	58.40 (51-64)	Cross-over, DB, PC	100µg/day E_2	Patch	3 days	Retention of story content, Sequential Memory task, Digit Ordering, Stroop	Immediate recall, sequential memory, working memory & response inhibition were better with ET.
Almeida et al (2006)	115	73.75 (>70)	R, DB, PC	2mg/day E_2	Oral	20 weeks	CAMCOG, Block Design, Memory for Faces, CVLT, Verbal Fluency	No effect.

Table 4. Continued…….

Study	N	M age (range)	Design	Treatment	Mode	Duration	Measures	Outcome
Dumas et al (2006)	15	60.40 (48-84)	Cross-over, R, DB, PC	1mg/day E_2	Oral	3 mths	CFF, CRT, DSST, divided attention task, continuous performance test, Selective Reminding Task, Verbal Paired Associates, Paragraph Recall	No effect.
Dumas et al (2008)	22	65.00 (50-81)	Cross-over, R, DB, PC	1mg/day E_2 for 1 mth then 2mg/day E_2 for 2 mths	Oral	3 mths	Selective Reminding Task, Paragraph Recall, CFF, CRT, Continuous Performance test	No effect.
Yaffe et al (2006)	417	66.75 (60-80)	R, DB, PC	14µg/day E_2	Patch	2 yrs	3MSE, Paired Associates, Visuospatial memory, Word List Recall, Trails B, modified Boston Naming Test, Verbal Fluency	No effect.
LeBlanc et al (2007) †	32	52.67 (NR)	R, DB, PC	2mg/day E_2	Oral	8 weeks	Verbal Paired Associates, Paragraph Recall, Memory for Faces (emotional memory), Visual reproduction, COWAT	No effect.
Maki et al (2007) †	180	52.15 (45-55)	R, DB, PC pilot	0.625mg/day CEE + 2.5mg/day MPA	Oral	4 mths	CVLT, Brief test of Attention, BVRT, Card Rotation test, COWAT, Paragraph Recall, Digit Span	No effect.
Pefanco et al (2007)	57	75.50 (≥ 65)	R, DB, PC	0.25mg/day E_2 OR + 100mg/day micronized progesterone (for 2 weeks every 6 mths)	Oral	3 yrs	COWAT, TMT, Wisconsin Card Sorting Test, Boston Naming Test, Digit Symbol Modalities Test, Complex Figure Test, Fuld Object Memory Test, Verbal Paired Associates	No effect.

Note: † included peri- and post-menopausal women. edu.- education, NS – not specified, DB- Double-Blind, R- randomized, PC- Placebo-Controlled, EB- Estradiol Benzoate, i.m- intramuscular, CEE- conjugated equine estrogens, T- Testosterone, DSST- Digit Symbol Substitution Test, MMSE- Mini-Mental Status Exam, 3MSE- modified Mini-Mental Status Exam, BVRT – Benton Visual Retention Test, CRT- Choice Reaction Time, MPA- Medroxy-progesterone acetate, TMT- Trail Making Task, cog- cognitive, SHBG- Sex Hormone-Binding Globulin, COWAT – Controlled Oral Word Association Test, CVLT- California Verbal Learning Test, PFC – prefrontal cortex, CAMCOG – Cambridge Cognitive Examination for Mental Disorders of the Elderly, CFF- Critical Flicker Fusion.

In further support of the theory that a progestagen may mitigate the positive effects of ET, is the well-know Women's Health Initiative Memory Study (WHIMS), a sub-study of the Women's Health Initiative Study (WHIS), which found long-term EPT to have an impairing effect on a measure of global cognition (3MSE) when compared to a placebo group (Rapp et al 2003b). However, this same group also found an impairing effect of ET on the 3MSE (Espeland et al 2004), suggesting that another factor may be responsible for their results (for example 'age', given that the WHIS involved women who were considerably older, aged 65-79). Similarly, the majority (72.7%) of experimental studies that used older women (*M* age between 60-82) did not find any significant ET-effects (Almeida et al 2006; Binder et al 2001; Dumas et al 2006; Dumas et al 2008; Goebel et al 1995; Janowsky et al 2000; Pefanco et al 2007; Wolf et al 1999; Yaffe et al 2006). Consistent with this, the studies that did find significant positive effects of ET comprised younger postmenopausal women, with the mean age ranging from 45-58 years (with the exception of Duka et al 2000). This brings into question the possibility of a 'critical period' after the menopause for which ET may be effective for enhancing cognitive functioning. Notably however, age and the addition of a progestagen are not the only factors that would account for inconsistencies, as some studies that also used younger postmenopausal women (*M* age between 47- 57) and administered estrogen only treatment, have failed to find significant effects on cognition (Ditkoff et al 1991; LeBlanc et al 2007; Polo-Kantola et al 1998; Rauramo et al 1975; Shaywitz et al 1999).

Another common feature among the studies with significant positive results is the type of estrogen used, with the majority (75%) administering E_2 treatment. This is not surprising as E_2 is the most potent of the estrogens and has been frequently found to act on neuronal systems at the molecular and cellular level, specifically influencing hippocampal and pre-frontal cortical cells and mechanisms (see Chapter 1 for review). Estrone (E_1), the main constituent of CEEs, has less frequently been shown to influence neuronal function, but some evidence for neuroprotection does exist (Bhavnani et al 2003; Kajta et al 2004; Regan and Guo 1997; Vedder et al 2000; Zemlyak et al 2002). Only one of the 6 experimental studies that administered CEEs found a significant positive effect where, after controlling for IQ, oral reading

improved for the ET group compared to the placebo group, however there were no effects on the 7 other cognitive measures used (Shaywitz et al 2003).

The 'type' of estrogen used is inherently related to the mode of administration and may also have an impact on the outcome. Transdermal preparations result in somewhat different plasma concentrations of circulating estrogens, as this mode bypasses hepatic metabolism and is absorbed into the bloodstream at a steady-state (Gleason et al 2005). Oral estrogens are thus metabolized much faster (by the liver and gut) and result in higher E_1 compared to E_2 levels and less bioavailability E_2 than with transdermal treatment (see Chapter 1, Table 2 for approximate serum E_2 and E_1 concentrations observed with different ET preparations in postmenopausal women). Despite this, as mentioned earlier, significant positive effects of ET on cognition have been observed with intramuscular (i.m), oral and transdermal preparations, although half of the 8 positive studies used the latter mode of administration (see Table 4 for details). Despite the findings of positive ET-effects, it should be acknowledged that 16 (61.5%) of the 26 studies failed to find any significant effects of ET on cognition, even when assessing multiple cognitive domains using tests that have previously been shown to be sensitive to ET (e.g Paired Associates and Paragraph Recall), and when using treatment durations that have also proven effective in the past (see Table 4).

2.3.6 Methodological Considerations for Experimental Studies

Of relevance is the duration of time passed before treatment is initiated after the transition to the post-menopause. The impact of this factor is difficult to ascertain, given that in the 8 studies that found significant effects, the average time since last menstrual period tended to vary in duration. Two studies comprised women with minimal delay as they received ET post-surgical menopause (Phillips and Sherwin 1992a; Sherwin 1988). Another stipulated time since last menstruation was <5 years (but they also included peri-menopausal women) (Joffe et al 2006). Two studies reported 3.25 and 9.1 mean years since last menstrual period (Krug et al 2003; Shaywitz et al 2003 respectively), while the remaining 2 studies did not report this statistic (Duka et al 2000; Wisniewski et al 2002). Average duration of time since

menopause onset also varied among the studies that failed to find significant ET-effects, ranging between approximately 1.75 years (Maki et al 2007) to 17.4 years (Wolf et al 1999). Some studies also had large variance between participants (e.g. 7-40 yrs: Wolf et al 1999). Rapp and colleagues (2003b) investigated whether the length of time before initiation of HT had an impact on results and found no difference between early, moderate and late initiators in respect to EPT-effects on cognition. The authors however, used a relatively insensitive cognitive measure (3MSE). Binder and colleagues (2001) used age of menopause onset as a covariate which also had no impact on results. It is also difficult to assess the influence of previous exposure to HT prior to participation in research. Although a few studies did report the number of participants who had received HT in the past (Almeida et al 2006; Binder et al 2001; Espeland et al 2004; Maki et al 2007; Rapp et al 2003b; Shaywitz et al 2003), only one analyzed this factor and discovered it had no impact on the study outcome (Rapp et al 2003b). In addition, Rapp and colleagues (2003b) found the duration of prior HT to be unrelated to the primary EPT-effects on cognition.

It is worth noting that some studies statistically controlled (or matched groups) for age (Dumas et al 2008; Hogervorst et al 1999; Sherwin 1988; Wisniewski et al 2002), education (Binder et al 2001; Sherwin 1988), pre-morbid IQ (LeBlanc et al 2007; Shaywitz et al 2003), socioeconomic status (Hogervorst et al 1999), age of onset of menopause (Binder et al 2001), type of menopause (Binder et al 2001), menopausal symptoms (Hogervorst et al 1999), baseline cognitive scores (Binder et al 2001; Maki et al 2007; Sherwin 1988), mood (Wolf et al 1999), multiple analyses/comparisons (LeBlanc et al 2007; Rapp et al 2003b) and practice effects (Krug et al 2003). Almost all experimental studies used a randomized double-blind placebo-controlled design, allowing for a natural distribution of individual variance between groups, with some even having the resources to utilize a cross-over protocol. Furthermore, the majority of studies (excluding those with a cross-over design) did not find any statistically significant baseline differences between the HT and placebo groups on most demographic variables in particular: age and education/baseline IQ (Almeida et al 2006; Binder et al 2001; Duka et al 2000; Espeland et al 2004; Joffe et al 2006; LeBlanc et al 2007; Pefanco et al 2007; Phillips

and Sherwin 1992a; Rapp et al 2003b; Shaywitz et al 2003; Wolf et al 1999; Yaffe et al 2006), ethnicity (Binder et al 2001; Espeland et al 2004; Joffe et al 2006; Phillips and Sherwin 1992a; Rapp et al 2003b; Yaffe et al 2006), socioeconomic status (Espeland et al 2004; Rapp et al 2003b), employment status (Joffe et al 2006), BMI (Espeland et al 2004; Rapp et al 2003b; Wolf et al 1999; Yaffe et al 2006), previous HT use (Almeida et al 2006; Binder et al 2001; Espeland et al 2004; Joffe et al 2006; Phillips and Sherwin 1992a; Rapp et al 2003b; Shaywitz et al 2003), type of menopause (Almeida et al 2006; Espeland et al 2004; Joffe et al 2006; Pefanco et al 2007; Phillips and Sherwin 1992a) and presence of menopausal symptoms (eg. hot flushes, fatigue, sleep disturbance etc.) (Joffe et al 2006; LeBlanc et al 2007; Phillips and Sherwin 1992a; Yaffe et al 2006).

A meta-analysis conducted in 2001 assessed 8 cohort studies and 9 experimental studies and concluded that HT generally enhanced certain cognitive process in women experiencing menopausal symptoms but had no apparent effect in asymptomatic women (LeBlanc et al 2001). In line with this Joffe and colleagues (2006) found that women with hot flushes at baseline showed greater improvements in verbal learning/memory after ET (although the ET-group as a whole also improved significantly). Improvement however, was only seen in one of the 14 cognitive variables. Recently LeBlanc and colleagues (2007) specifically tested the effect of menopausal symptoms on cognition. It was found that women with high level symptoms (despite having significantly poorer sleep and more negative mood), performed no differently to low symptom women on cognitive tasks. In addition, while ET significantly improved symptoms as well as sleep relative to the placebo group, there was no change in cognition over the 8 weeks of the study.

Lastly, it should be noted that some studies only included women with surgical menopause (ie. abdominal hysterectomy/bilateral salpingo-oophorectomy) (Almeida et al 2006; Ditkoff et al 1991; Espeland et al 2004; Phillips and Sherwin 1992a; Rauramo et al 1975), given that these participants would be more significantly deprived of endogenous estrogens, as opposed to women with natural menopausal who have intact ovaries (which can still produce very low levels of endogenous

estrogens <150pmol/L) (Leonard 2004). Inferences from these studies are thus limited to a subgroup of the post-menopausal population.

2.3.7 Estrogen Treatment in Clinical Samples

Inferences of the cognitive effects of estrogens can also be drawn from experimental trials in clinical cohorts. A small handful of studies have been conducted in young women with genetic abnormalities leading to compromised HPA axis functioning. Women with uterine myomas are typically treated with gonadatropin releasing hormone (GnRH) agonists, which will ultimately lead to a hyopestrogenic state. Sherwin and Tulandi (1996) found that women (M age = 34.2) with this condition who were given 8 weeks of CEE in addition to a GnRH agonist (which had been initiated 12 weeks prior), improved significantly on measures of verbal memory when compared to the placebo group. Grinspoon and colleagues (2003) gave a combined oral contraceptive (a progestagen + ethinyl estradiol) to young women with hypothalamic ammhenorhea for 84 days under a randomized double-blind placebo-controled design and found no effects on cognition. Another study in children with Turner's Syndrome (a condition characterized by an inability of the ovaries to produce estrogen) found that an average of 4 years treatment with ethinyl estradiol significantly improved performance and speed of information processing during visual-motor and visuo-spatial tasks (Ross et al 1998). Research involving postmenopausal women with diagnosed medical conditions also provides some insight into the effects on ET on cognition. Grady and colleagues (2002) found that women with established coronary disease, who had received EPT for a mean of 4.2 years, performed significantly worse than the placebo group on a verbal fluency test and a word list memory test. An EEG study by Saletu (2003) involved postmenopausal women with a diagnosis of insomnia, who received HT for 2 months. They reported significant improvements in speed of information processing, as inferred from P300 latency (no other cognitive tasks were administered). Collectively, these studies provide support for the cognitive-enhancing effects of ET, with EPT appearing to be the less reliable treatment option. Despite these few studies, the clinical cohort most frequently examined in terms of ET-effects on

cognition is women with AD, given the implications this may have on the neurodegenerative process and treatment of cognitive deficits.

While there is sufficient support for HT-use reducing the risk of developing AD in later life (Costa et al 1999; Paganini-Hill and Henderson 1996; Tang et al 1996; Waring et al 1999; Zandi et al 2002), few controlled experimental trials have found estrogen useful in treating this illness. An early randomized controlled trial (RCT) of CEE for 3 weeks in 14 women suffering AD showed an improvement in one of three dementia scales (Honjo et al 1993; In Sherwin 2003). These findings are questionable as some women were also administered a progestagen during the 3 weeks. Asthana and colleagues (1999) conducted a double-blind placebo-controlled pilot study in 12 women with probable dementia and found that 50µg/day of transdermal E_2 for 8 weeks significantly improved performance on tasks of attention and verbal memory. This study was replicated using a 100µg/day dose in 20 patients and significant improvements were again seen in these same cognitive domains as well as visual memory and semantic memory (Asthana et al 2001). Despite these positive findings, more recent larger-scale studies have failed to find significant cognitive-enhancing effects with varying doses of CEE of longer treatment durations (ranging 12 weeks - 15 months) (Henderson et al 2000; Mulnard et al 2000; Wang et al 2000; Yoon et al 2003). It may be that transdermal E_2 is more effective at the cellular level in the brain and may therefore be more likely to elicit appreciable improvements in cognitive functioning than CEEs. Future research should examine whether a longer duration of transdermal E_2 treatment would be effective in improving cognitive deficits in a larger sample of AD patients. A broad body of literature on HT and AD exists, yet a full review of this literature is beyond the scope of the present thesis (for a full discussion of this literature please see the following reviews Brinton 2001; Fillit 2002; Miller et al 2001; Pinkerton and Henderson 2005; Sano 2000).

2.4 Summary of Human Studies

Menstrual cycle and correlation research has provided some support for estrogen as a modulator of cognition. However, this area of research is quite variable given the

short window during which endogenous estrogen levels remain stable before entering the next phase of the cycle. Therefore, research in this area is somewhat unreliable, and opposing effects have been found on a number of cognitive domains including verbal fluency, spatial memory and implicit memory. The implication that peripheral measures of plasma estrogens do not necessarily reflect estrogen levels in the brain, may account for such discrepancies and needs to be taken into consideration when interpreting this literature. Observational and epidemiological research, while extremely mixed, provides the greatest support for the estrogen protection hypothesis. This hypothesis postulates that HT-users demonstrate a clear advantage over non-users on numerous cognitive tests, in particular measures of verbal fluency and verbal and visual memory. Some evidence for preservation, and in some cases enhancement, of certain cognitive processes also exists in prospective research of long-term ET/HT. Despite attempts to statistically control for typical confounding variables such as age and education, this literature is rife with methodological inconsistencies where factors such as duration of treatment, consistency of usage, dose and type of estrogen administered remain out of the researcher's control and thus vary greatly within and between studies. More reliable assessments of ET-effects on cognition can be drawn from experimental trials, yet the majority of RCTs did not find significant results. Nevertheless, some studies do support that of previous research with verbal learning/memory being the domain to most frequently improve with ET. A lack of significant findings may be due to the age at which participants enter the trial, the addition of a progestagen, the type of estrogen used (ie. E_2 vs CEE), the route of administration, or a combination of these factors.

Lastly, while at present there is insufficient evidence to suggest ET is effective in treating the cognitive deficits associated with AD, only a handful of studies have been conducted. Of the 6 studies, two reported significant enhancing effects. Incidentally, these were the only two studies that use transdermal E_2, thus further research is needed in this area. Given the proposal of adjunctive estrogen as a possible treatment option for disorders characterized by cognitive deficits, including schizophrenia, it is important to determine whether cognitive effects of ET are specific to post-menopausal women or whether healthy young women with normal endogenous levels of estrogen would also benefit from additional ET. This would

further help to understand the magnitude of estrogen's effects on cognition and possibly reveal predisposing factors that may be required for significant ET-effects. It may be that, for ET to influence performance there must be (1) a substantial decline in cognitive functioning and/or (2) a substantial reduction in endogenous estrogen levels, such as those seen with increasing age and the menopause. To our knowledge, this has never been explored.

Chapter 3
Brief Overview of the Cholinergic System in Cognitive Function and the Modulatory Role of Estrogens

Chapters 1 and 2 outlined the theoretical foundations for choosing to investigate the effect of estrogen on cognition. This Chapter will now give a brief overview of the cholinergic system and outline the reasons for exploring this system as a possible mechanism via which estrogen may modulate certain cognitive processes.

3.1 The Cholinergic System

The neurotransmitter acetylcholine (ACh) was first discovered in the 1920's and has since been acknowledged as a fundamental neurochemical, having numerous roles within the central as well as the peripheral nervous systems. The precursors choline and acetyl-coenzyme A, are synthesized by the enzyme choline acetyltransferase (ChAT) to form ACh, which is then stored in pre-synaptic vesicles (Sarter and Parikh 2005). Once released into the synaptic cleft, ACh binds to multiple muscarinic (G-protein coupled or metabotrophic, i.e. M1-M4) receptors and nicotinic (ligand-gated ion channel or ionotrophic) receptors (i.e. $\alpha4\beta2$, $\alpha7$, etc). Free ACh is cleared from the synaptic cleft via the enzyme acetylcholine esterase (AChE), which converts ACh into the inactive metabolites 'choline' and 'acetate'. There are two locations in the brain that are primarily, although not entirely, dominated by cholinergic neurons (1); the basal forebrain (BF) which consists of the medial septum (MS), diagonal band of Broca (dbB) and nucleus basalis magnocellularis (NBM) (also called nucleus Basalis of Meynert), and (2); the brain stem which includes the pedunculopontine tegmental nucleus and the latero-dorsal pontine tegmentum (Mesulam et al 1983a) (see Figure 6 for diagram of cholinergic system and projection pathways). Because BF cholinergic neurons are interspersed with non-cholinergic neurons, Mesulam and colleagues (1983a; 1983b) categorized these cholinergic cells into 4 different groups (Ch1-4) based on the location they originate from and the regions they project to. 'Ch1' refers to the cholinergic neurons in the medial septum which project predominately to the hippocampal complex as do the 'Ch2' neurons, which originate in the vertical dbB. The 'Ch3' group comprise neurones in the horizontal dbB and project primarily to the olfactory bulbs, while 'Ch4' neurons originate in the NBM and essentially innervate the cerebral cortex and amygdala (Mesulam et al 1983a; Mesulam et al 1983b). Approximately one third of the Ch4 neurones express the M2 muscarinic receptor subtype, which appears to be the more dominant cholinergic

receptor of the NBM, while the M1 subtype dominates cholinergic transmission in the cerebral cortex (Mesulam 1996). Hence, the BF cholinergic neurons are the major source of cholinergic input to the cortex and the hippocampal formation, implicating this neurotransmitter system in an array of fundamental cognitive processes (for review of cholinergic neuronal networks see Lucas-Meunier et al 2003).

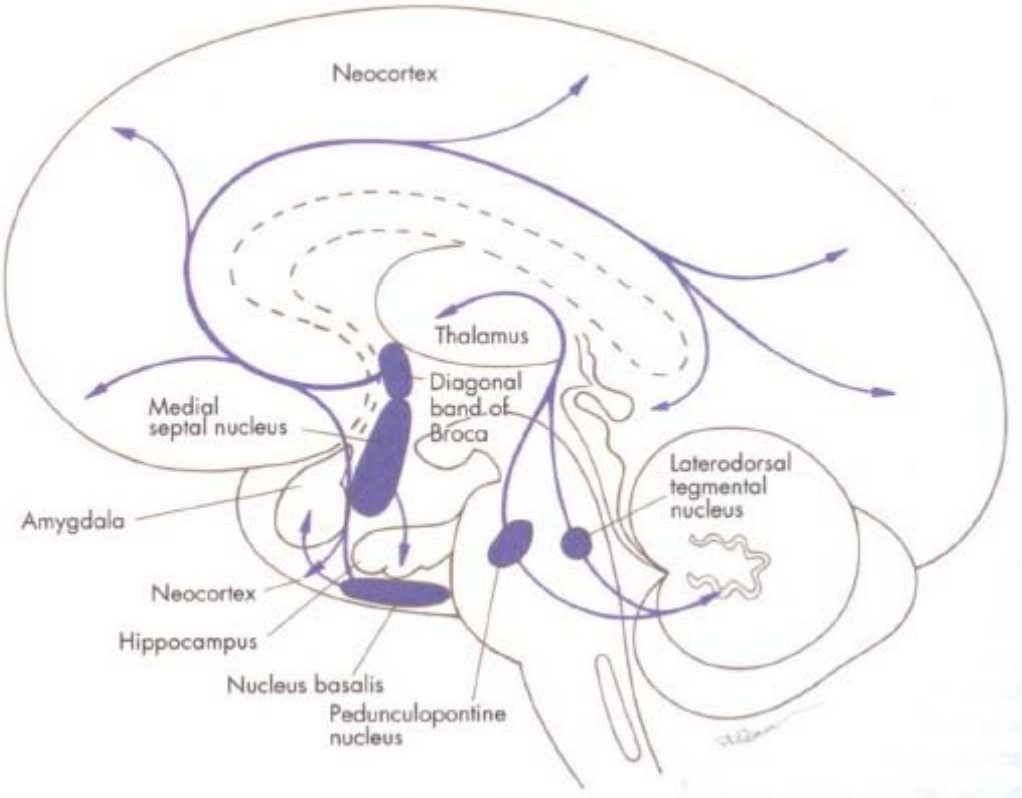

Figure 6. The Cholinergic System and Projection Pathways *(adopted from Schatzberg and Nemeroff 2004).*

3.2 Cognitive Domains Reliant on Cholinergic Functioning

Up until the late 80s the cholinergic system had long been acknowledged as the foundation of almost all learning and memory processes. However, in the early 90s the development of the neurotoxin 192 IgG-saporin (SAP) caused a stir in the research field, as this selective ribosome inactivating neurotoxin specifically destroys

cholinergic neurons (bearing NGF-receptors) while sparing the non-cholinergic neurones of the BF (Schliebs et al 1996). This brought into question findings from earlier lesion studies that used non-selective neurotoxins such as NMDA receptor agonists, which produced impairments in numerous cognitive functions (for review see Dunnett et al 1991). Since the utilisation of SAP, there has been some debate as to which cognitive processes are impaired following cholinergic cell loss, with some reviewers concluding the cholinergic system is predominantly involved in attentional processing and short-term spatial (working) memory but not in learning and memory per se (Everitt and Robbins 1997). However, this speculation has since been challenged, as recent studies have shown significant impairments in acquisition of DMP and associative learning tasks following selective MS and vertical dbB SAP-induced lesions in both rats (Fitz et al 2006; Janisiewicz et al 2004; Johnson et al 2002) and monkeys (Fine et al 1997; Ridley et al 2005; Ridley et al 1999). Another study using tetrodotoxin (a reversible sodium channel blocker) infused into the NBM also showed that the BF cholinergic system was required for the acquisition of a conditioned taste aversion task, but not the retrieval/recall of the aversive memories (Miranda and Bermudez-Rattoni 1999). In support of this, levels of extracellular ACh in the hippocampus and parietal cortex have been found elevated during acquisition of an operant behaviour task (i.e. learning) but not during memory recall (Orsetti et al 1996). In contrast, although Pizzo and colleagues (2002) found SAP-induced lesions caused impairments in acquisition of water and T-maze tasks, they also surprisingly found memory was impaired on an inhibitory avoidance task. Pizzo and colleagues (2002) suggested that observed impairments in learning and memory is dependent on the route of SAP administration (ie. intracerebrao-ventricular vs. intraparenchymal) and therefore the extent of lesioning to the MS and NBM, as well as the complexity of the task (Pizzo et al 2002). Recent findings support this hypothesis and further suggest that lesioning of the MS/NBM may result in learning deficits due to the response strategy adopted, which appeared to be that involving egocentric as opposed to place/allocentric response patterns (Gibbs and Johnson 2007).

Similarly, although research generally supports a major role of the MS/vertical dbB cholinergic neurons in spatial working memory (Janis et al 1998; Leanza et al 1996;

Shen et al 1996; Walsh et al 1996), some researchers have failed to find spatial working memory impairments following SAP lesioning of these regions (Chappell et al 1998; Frielingsdorf et al 2006; Kirby and Rawlins 2003; McMahan et al 1997; Pang and Nocera 1999). These mixed findings are again possibly due to the method of SAP administration and the inconsistent targeting of different BF nuclei, as more extensive lesioning beyond just the MS/vertical dbB (ie. the NBM) may be required for significant impairments in spatial working memory (Wrenn et al 1999). While there is currently some controversy surrounding the conditions under which the cholinergic system plays a significant role in learning and memory, there is little debate over its role in attention and sensory information processing.

The basal forebrain corticopetal cholinergic projections, including those to the limbic system, are acknowledged as having an essential role in attentional processing as evidenced by: (1) impaired performance following selective BF cholinergic lesions, (2) increased cortical ACh release during attentional tasks, and (3) increased cortical activity and improved performance with muscarinic/nicotinic receptor agonists (for reviews see Everitt and Robbins 1997; Sarter et al 2003; Sarter et al 2005a). Two interacting mechanisms have been proposed to govern cholinergic cortical activation: signal-driven modulation of detection (increases in attentional effort in response to increased task demand) and top-down modulation of detection (pre-frontal modulation of cholinergic inputs via knowledge-based filtering of irrelevant stimuli) (Sarter et al 2005a). More recently the role of the cortical cholinergic system in attentional functions has been linked to the regulation of sensory input processing, given that; (1) dual activation of muscarinic receptors and thalamic inputs mediates cortical sensory plasticity, (2) cortical ACh selectively enhances thalamocortical inputs while suppressing associational inputs, and (3) stimulation of cholinergic receptors increases the cortical representation of sensory stimuli (for review see Sarter et al 2005a). In summary, there is ample evidence to support the cholinergic system as having a significant fundamental role in attentional processes.

Insight into the role of the cholinergic neurotransmitter system in cognitive processes was not only extrapolated from basic science research but clinical based research as well. Due to the extensive cholinergic cell loss in AD patients and the subsequent

impairments in cognitive functioning, researchers adopted the 'scolopamine model' in particular, to test the effects of potential pharmacological treatments designed for AD and other disorders characterised by cognitive deficits.

3.3 The Scopolamine Model

Scopolamine, a mucarinic receptor antagonist, has long been a reliable pharmacological tool used as a cognitive model in both animals and humans (for review see Ebert and Kirch 1998; Hasselmo and Wyble 1997; Patel and Tariot 1991). Scopolamine acts by reducing the effectiveness of acetylcholine at the site of the synapse by binding (non-selectively) to muscarinic receptors on the post-synaptic terminal, but without causing depolarization (Deutsch 1971). Scopolamine, also known as hyoscine hydrobromide, is a belladonna alkaloid drug derived from plants of the Solanaceae family. The oral and transdermal forms are commonly used to treat motion sickness, while the injectable form is often used as a pre-operative sedative. Central pharmacodynamic effects of scopolamine are reported to peak between 1 and 3 hours and are eliminated within 5-6 hours (Ali-Melkkila et al 1993). Given the fast-acting transient effects of scopolamine, it has proven useful and convenient in testing the acute effects of muscarinic receptor blockade on cognitive function in healthy human subjects. Early research consistently showed word list learning and free recall to be significantly impaired while recognition memory was unaffected post-scopolamine (Crow and Grove-White 1973; Ghoneim and Mewaldt 1975; Ghoneim and Mewaldt 1977; Mewaldt and Ghoneim 1979; Petersen 1977). Furthermore, findings from these studies suggested that scopolamine impaired the encoding of new information specifically, given that the word lists learnt prior to scopolamine administration were able to be freely recalled while recall of the lists learnt post-scopolamine administration were severely impaired, which is in line with the hippocampal model of learning and memory. Scopolamine has also been shown to significantly impair performance on paired associate tasks, measures of learning and memory (Caine et al 1981; Crow and Grove-White 1973; Ostfeld and Aruguete 1962), which further supports a modulatory role of cholinergic hippocampal neurons in specific cognitive processes.

Consistent with early findings subsequent studies have also shown scopolamine to induce significant impairments in not only hippocampal-mediated processes but a wide range of cognitive functions including: verbal fluency and oral reading, verbal learning and memory, working memory, visuospatial memory, logical reasoning, sustained attention and information processing/psychomotor speed (Aarsland et al 1994; Brandeis et al 1992; Broks et al 1988; Ebert et al 1998a; Edginton and Rusted 2003; Ellis et al 2006; Erskine et al 2004; Flicker et al 1990; Green et al 2005; Koller et al 2003; Little et al 1998; Patat et al 1991; Robbins et al 1997; Rosier et al 1998; Rusted and Eaton-Williams 1991; Rusted 1988; Rusted et al 1991; Rusted and Warburton 1988; Sloan et al 1992; Sperling et al 2002; Wesnes and Warburton 1984). While some of these studies suggested a dose-dependent effect of scopolamine on performance (Aarsland et al 1994; Broks et al 1988; Koller et al 2003; Robbins et al 1997; Wesnes and Warburton 1984), the majority found significant impairments with varying doses (ranging from 0.2mg to 0.65mg) and with both oral, subcutaneous and intramuscular administration.

Quantitative electroencephalogram (EEG) studies in healthy individuals also provide evidence for scopolamine as a good model for the cognitive deficits observed in AD. Specifically, muscarinic receptor blockade with scopolamine has been shown to increase delta and theta frequency bands while decreasing alpha and beta frequency bands, implying decreased brain activity (for review see Ebert and Kirch 1998; Ebert et al 1998b; Kikuchi et al 1999). Moreover, these scopolamine-induced changes in EEG activity are similar to the EEG recordings of patients with AD and senile dementia (Elmstahl et al 1994; Neufeld et al 1994; Rosen et al 1993). In addition, the visual and auditory P3 event-related potential has been shown to have a decreased amplitude following scopolamine, with evidence to also suggest decreased P3 amplitude in the frontal but not parietal area, which was found to be associated with concurrent impairment in recognition memory performance (Potter et al 2000). Imaging research has also found scopolamine-induced reductions in regional cerebral blood flow (rCBF) of the prefrontal cortex (PFC) and anterior cingulate during a verbal memory task (Grasby et al 1995). An fMRI study found marked decreases in both the extent and magnitude of activation of the hippocampus, inferior PFC and fusiform gyrus following scopolamine administration and whilst participants

performed a novel visual paired-associates task (Sperling et al 2002). In conjunction with this finding, post-scan memory testing of the paired-associates task was significantly impaired, particularly on the free recall measure for names, suggesting encoding of novel face-name pairs requires intact muscarinic cholinergic receptors. Similar results were found by Schon and colleagues (2005) using fMRI and a delayed match-to-sample task to test scopolamine-induced changes in parahippocampal activity (reportedly related to long-term encoding).

The scopolamine model has been utilised to test the efficacy of a number of cholinergic enhancing drugs. A single dose (2mg) of physostigmine (a cholinesterase inhibitor) administered 90 minutes post-scopolamine, has been shown to significantly reverse the impairing effects of scopolamine on measures of verbal memory, sustained attention, spatial working memory, information processing/ psychomotor function, and recognition memory, in healthy males (Ebert et al 1998a). A double-blind placebo-controlled cross-over study in healthy elderly volunteers similarly found donepezil and ZT-1 (a precursor of the novel cholinesterase inhibitor huperzine) both significantly reversed the effects of scopolamine on measures of attention, working memory and episodic memory (Zangara et al 2004). Acute doses of the cholinesterase inhibitor velnacrine, have also been shown to repeatedly reverse cognitive impairment induced by scopolamine in healthy volunteers as well as improve recognition memory in AD patients (for review see Siegfried 1993). These findings support animal research that has consistently shown cholinergic agents such as tacrine, donepezil, physostigmine, metrifonate and galanthamine, to reverse the scopolamine-induced impairments in attention, learning, short-term memory and spatial working memory (Braida et al 1998; Buccafusco et al 2007; Chen et al 2002; Chopin and Briley 1992; Kirkby et al 1996; Lindner et al 2006; Rupniak et al 1990; Rupniak et al 1997; van der Staay and Bouger 2005; Yamaguchi et al 2001). The consistent finding of scopolamine-induced impairments in numerous cognitive processes and the relationship with decreased activation in the frontal cortex, hippocampal and parahippocampal structures, together with research showing reversal of cognitive impairments with cholinergic drugs, provides strong evidence for this model as a reliable tool for testing the effects of cholinergic agents on

cognition (for information on other pharmacological models of cognition see Gilles and Luthringer 2007).

In summary, there is undeniable evidence that the cholinergic system and its projections to the frontal cortex, hippocampus and limbic system play an important role in the execution of specific cognitive functions, namely verbal and visual learning, memory consolidation, declarative and spatial memory, working memory attention and information processing, which is widely acknowledged in the literature (for further information on the role of the cholinergic system in cognition see reviews by Baxter and Chiba 1999; Everitt and Robbins 1997; Freo et al 2002; Power et al 2003; Riedel and Jolles 1996; Sarter et al 2005a). As it is well-known, a number of clinical disorders are characterised by cholinergic cell loss or dysfunction and although AD and Parkinson's Disease are most commonly associated with cholinergic dysfunction, such abnormalities have also been observed in a number of other disorders, including schizophrenia. This chapter will now review cholinergic system dysfunction in schizophrenia, with a focus on muscarinic receptors.

3.4 Cholinergic Dysfunction in Schizophrenia

As described above the cholinergic system plays a fundamental role in numerous cognitive processes. Thus, it is not surprising that the cognitive impairments inherent to schizophrenia are predicted to predominantly arise from abnormalities in the integrity and regulation of the basal forebrain cholinergic system and projection pathways. Notably however, the classical cholinergic neuropathology observed in AD is not apparent in schizophrenia patients, given there are no discernible differences in cholinergic markers (Haroutunian et al 1994; Powchik et al 1998) or in the size or number of cholinergic cells (el-Mallakh et al 1991) in the brains of schizophrenia patients when compared to normal controls. Despite this, ChAT activity was found to be significantly negatively correlated with antemortem clinical dementia rating scores in schizophrenia patients (Powchik et al 1998). Other post-mortem studies using muscarinic receptor antagonists such as [^{3}H]QNB, [^{3}H]pirenzipine and [^{3}H]AF-DX 384, have shown reduced muscarinic receptor binding in the striatum, hippocampus, dendate gyrus, cingulate, frontal, parietal and

temporal cortices of schizophrenia patients (Bennett et al 1979; Crook et al 1999; Crook et al 2000; Crook et al 2001; Dean et al 1996; Dean et al 2002; Newell et al 2007; Scarr et al 2007; Zavitsanou et al 2004). Interestingly, Zavitsanou and colleagues (2004) found decreased muscarinic M1 and M4 receptor binding in the anterior cingulate cortex to be specific to schizophrenia patients when compared to patients with depression and bipolar affective disorder. However, a more recent study found no change in M_2 and M_4 receptor binding in the anterior cingulate of either schizophrenia, depression or bipolar patients (Zavitsanou et al 2005). Collectively, findings suggest a tendency towards a subtype-specific reduction in M1 receptors in specific brain regions. The findings of decreased radioligand binding have been suggested to reflect down-regulation of muscarinc receptors either in response to increased ACh efflux or a primary alteration in receptor transcription. This later hypothesis is supported by findings of significantly decreased levels of muscarinic M1 mRNA in the frontal cortex (Mancama et al 2003), but not in the caudate-putamen (Dean et al 2000), further supporting regions-specific alterations in cholinergic muscarinic receptor functioning.

It has been suggested that findings of dysregulation of the cholinergic system are likely due to the anticholinergicity of many atypical antipsychotic medications (such as clozapine and olanzapine), given that anticholinergic load has been found associated with poorer performance on measures of attention, as well as declarative visual and verbal memory (Minzenberg et al 2004; Tracy et al 2001). Furthermore, research using *in vivo* single photon emission computed tomography (SPECT) has shown reduced muscarinic receptor availability in patients administered clozapine (Raedler et al 2003a) and olanzapine (Lavalaye et al 2001; Raedler et al 2000). Thus, given the findings presented above are drawn from post-mortem studies it is at this stage difficult to know whether cholinergic dysregulation is inherent to the pathogenesis of schizophrenia or whether it is a result of long-term use of antipsychotic medications. To our knowledge there is only one study that has investigated muscarinic receptor density in vivo in medication-free patients, using SPECT and iodinated quinuclidinyl benzilate ([I-123]IQNB) as the ligand (Raedler et al 2003b). Patients had been medication-free for an average of 18 days and showed a significant reduction in muscarinic receptor availability in the frontal,

temporal and occipital corticies as well as the caudate, putamen and basal ganglia when compared to matched controls, suggesting alterations in cholinergic muscarinic functioning may be inherent to the pathophysiology of schizophrenia.

Based on the above findings cholinergic agents have been proposed as possible treatment options for the cognitive deficits seen in schizophrenia, which will be reviewed later in chapter 6 (for further review see Ferreri et al 2006; Friedman 2004). It should be taken into consideration that the cognitive impairment (along with the pathophysiology of schizophrenia) is most likely due to a combination of multiple dysfunctional neurotransmitter systems, and it may be the interactions between the dysfunctional cholinergic, dopaminergic and GABAergic systems which consequently leads to impairments in cognitive functioning (for more information see Sarter et al 2005b). This chapter will now explore the evidence supporting the neuroprotective effects of E_2 on the cholinergic system with evidence for both pre and post-synaptic interactions between E_2 and cholinergic mechanisms.

3.5 Estrogen and the Cholinergic System: Neurochemical Evidence

3.5.1 Effects of Estrogens on Choline Acetyltransferase

Of all the neurotransmitter systems the cholinergic system has been most frequently associated with the modulatory mechanisms of E_2. Research has found ChAT mRNA levels in certain brain regions (MS and striatum) to fluctuate with the estrous cycle, where higher ChAT mRNA levels were observed during diestrus (low estrogen phase) (Gibbs 1996). Findings may be due to a delay-dependent effect of estrogen on ChAT mRNA expression, given that an increase in ChAT mRNA expression was detected in the MS 24, 53 and 72 hours after an acute dose of 10μg E_2, but not as soon as 5 hours post E_2 administration (Gibbs 1996). In addition, Gibbs (1998) found that ChAT mRNA decreased in the MS and NBM after 6 months post-ovariectomy but not 3 months post-ovariectomy. This suggests that there may be a transient period where endogenous estrogens are neuroprotective for BF cholinergic neurons even after production of endogenous estrogens have ceased. Furthermore, three days of E_2 treatment administered at the 6-month time point partially restored ChAT mRNA levels in the MS (Gibbs 1998). These findings are supported by research showing

decreases in ChAT mRNA levels in the MS, NBM, hippocampus, striatum and frontal cortex following ovariectomy (Gibbs et al 1994; Heikkinen et al 2002; McMillan et al 2002; McMillan et al 1996; Singh et al 1994), which were seemingly restored with E_2 or tamoxifen (a selective estrogen receptor modulator; SERM) treatment (Gibbs et al 1994; McMillan et al 2002; McMillan et al 1996; Singh et al 1994), thus a certain level of E_2 in the brain may be required for normal cholinergic functioning. Furthermore, OVX female rats given E_2, EB, tamoxifen, raloxifene (also a SERM) or soy phytoestrogen treatment have demonstrated increased ChAT activity in numerous brain regions including the NBM, dbB, olfactory bulbs, hippocampus and frontal cortex (Gibbs 2000a; Lapchak et al 1990; Luine 1985; Pan et al 1999; Singer et al 1998; Wu et al 1999), suggesting a neuroprotective effect of E_2 on cholinergic neurons in key brain regions essential for cognitive functioning. However, the modulatory role of estrogens in these brain regions may vary as Lapchak and colleagues (1990) found chronic E_2 treatment to increase the spontaneous release of ACh from cultured hippocampal slices but not cortical slices. Gibbs and colleagues (2002) in a later study failed to find an increase in ChAT activity following 2 years chronic CEE treatment in OVX female cynomolgous monkeys. This may have been due to the use of this type of ET, which as discussed in Chapter 2, appears to be the least effective form of ET in terms of preservation or enhancement of cognitive performance. Gibbs and colleagues (2002) also found that the addition of a progestagen (MPA) to the ET actually resulted in a significant decrease in ChAT activity in the MS/bdB, further supporting the cognitive behavioural literature.

Choline acetyl transferase immunoreactivity (ChAT-IR) (i.e the staining of ChAT protein) has also been utilised to investigate estrogens' effects on cholinergic functioning. Gibbs and Pfaff (1992) discovered a significant (28.2%) increase in ChAT-IR cells in the MS of E_2-treated female rats on day 7 of treatment. However, this effect had dissipated at 30 days of treatment, implying that cholinergic cells may normalize by down-regulating protein-production after initial upregulation, possibly via biofeedback mechanisms. Interestingly, this relationship and time-course varied between BF regions, with the vertical dbB showing an increase after 14 and 30 days of treatment while the horizontal dbB showed a decrease in ChAT-IR after 7 days but

not after 14 or 30 days of treatment. Gibbs (1997) later found a duration and dose-dependant effect where 2, 10 and 25μg of E_2 for one week resulted in an increasing number of ChAT-IR cells in the MS, while 100μg had no effect, suggesting an inverted U-shaped response. Again, ChAT-IR levels in the MS had increased at 7 days of treatment but not when given for longer durations (2 or 4 weeks) (Gibbs 1997). A significant increase in ChAT-IR was also observed in the NBM, but only when the 10μg dose was administered for 1 week or after the 2μg dose was administered for 2 weeks (Gibbs 1997). Since then research has shown ovariectomy to result in significant decreases in ChAT-IR in the MS, dbB, NBM (Mufson et al 1999) and substantia innominata (Yamamoto et al 2007) of rodents, as well as the PFC (Kritzer and Kohama 1999) and vertical dbB (Kompoliti et al 2004) of Rhesus monkeys. Estradiol treatment was also shown to restore ChAT-IR in two of these studies, in that levels were comparable to intact-controls (Kritzer and Kohama 1999; Yamamoto et al 2007). Conversely, a study by Kalesnykas and colleagues (2004) showed no difference in the number of ChAT-IR cells between E_2-treated and vehicle-treated mice. This may have been due to the sample comprising aged mice or that C57BL/6J mice were used, as ChAT-IR can vary between mice strains (Schwegler et al 1996).

Research using combined autoradiography and immunostaining techniques has produced further evidence linking estrogen to the BF cholinergic system. Toran-Allerand and colleagues (1992) found that many of the neurons in the BF which were positive for the radiolabeled estrogen 17α-iodovinyl-11β-methoxyestradiol (^{125}I-estrogen; which has a similar binding affinity for both ERα and ERβ), also co-expressed ChAT mRNA or ChAT-IR. Shughrue and colleagues (2000) found 41%, 32%, 29% and 4% of cholinergic neurons in the MS, vertical dbB, horizontal dbB and the NBM respectively, contained ERs. In addition, the vast majority of BF neurons that contained ERs were ChAT immunoreactive irrespective of whether or not they were cholinergic neurons (Shughrue et al 2000). Miettinen and colleagues (2002) found similar results where approximately 60% of cholinergic neurons in the MS/vertical dbB, 46% in the horizontal dbB and 14% in the NBM co-expressed ERα and ChAT-IR. In contrast, two monkey studies have failed to demonstrate co-localization of ERα with ChAT-IR within any region of the BF (Blurton-Jones et al

1999; Perez et al 2004). Comparisons between intact and OVX-animals have revealed mixed findings as research has shown OVX to be associated with significant decreases (Mufson et al 1999), significant increases (Kalesnykas et al 2004) an no change (Kalesnykas et al 2005), in the percentage of double-labelled (i.e. ERα and ChAT) cholinergic BF neurons.

3.5.2 Effects of Estrogen on Choline Uptake, VAChT Density and ACh Release

High-affinity choline uptake (HACU), that is the rate at which choline (a precursor of acetyl choline synthesis) is taken up by the pre-synaptic neuron, has been found to increase in the cerebral cortex, frontal cortex, hippocampus and olfactory bulbs of rats after short-term (3 days - 5 weeks) treatment with E_2, E_2 benzoate (EB), EB plus progesterone or progesterone alone, relative to OVX control rats (Gibbs 2000a; O'Malley et al 1987; Singh et al 1994). Increases in HACU results in more choline readily available for synthesis into ACh, thus ovarian hormones may contribute to more efficient cholinergic functioning in these brain regions. Interestingly, Singh and colleagues (1994) found the E_2-treatment effects on HACU to coincide with improved performance on an active avoidance task. This further supports E_2 as a positive modulator of cholinergic functioning and learning. However, like ChAT-IR, increases in HACU may depend on the hormonal regimen used and the duration of treatment, as continuous long-term E_2 treatment (13 months) has resulted in a decrease in HACU in the hippocampus when compared to OVX controls (Gibbs 2000a).

Like ChAT-IR, vesicular acetylcholine transporter (VAChT) immunoreactive neurons in the dorsal hippocampus have been found to co-express extranuclear ERα-IR on presynaptic terminals (Towart et al 2003), implying estrogen may affect cholinergic functioning via a direct effect on local ACh release or uptake. Binding density of the VAChT has also been found to be influenced by ET. Specifically, OVX cynomolgus monkeys treated with placebo for years had significantly decreased VAChT immunopositive fibre density compared to CEE-treated and intact monkeys. However, this was only found in layer II of PFC as no differences were observed between treatment groups for the five other cortical layers or the parietal

cortex. Furthermore, no group differences were found in VAChT-positive neuron numbers or volume within the NBM, suggesting that ET has little effect on VAChT density (Tinkler et al 2004). Future research should investigate the effects of E_2 treatment on VAChT fibre density, given that this estrogen has more consistently been shown to have neuroprotective effects.

In vivo microdialysis techniques have been utilised to examine the effects of ET on ACh release, given that past research has linked hippocampal ACh output to better performance on learning and memory tasks (Gold 2003). Marriott and Korol (2003) found OVX rats injected with 10µg E_2 at 48 and 24 hours prior to testing, displayed a significant increase in hippocampal ACh release during the 'training phase' of a place-learning task, but not when at rest or during the recall phase of the task. Similarly, research has found increases in potassium-stimulated ACh release in the hippocampus and overlying cortex of OVX rats following short-term (11-30 days) E_2 treatment or high dose raloxifene (Gibbs et al 2004; Gibbs et al 1997). Interestingly, Gibbs and colleagues (2004) found that E_2 but not raloxifene treatment, significantly enhanced acquisition of a spatial DMP task in these animals prior to microdialysis. This further suggests E_2 is the more reliable form of ET which is more likely to elicit behavioural and neurochemical responses.

3.5.3 Effects of Estrogen on Acetylcholine Esterase Activity

Research on the effects of ET on AChE activity is limited. Monkey research has shown 2 years EPT (CEE plus MPA) significantly decreased AChE activity in the MS/dbB compared to OVX controls (Gibbs et al 2002). Similar findings have been observed in the entorhinal, insular and cingulate cortecies of aged monkeys following 3 weeks E_2-cypionate treatment (Kompoliti et al 2004). A study in rodents found OVX rats given E_2 dipropionate (EDP) demonstrated lower AChE activity in the frontal cortex as compared to OVX controls (Das et al 2002). Collectively, findings imply enhanced cholinergic functioning in brain regions essential for cognitive processing. Incidentally, Das and colleagues (2002) also found AChE activity was higher in the hippocampus and striatum of EDP-treated rats in comparison to the OVX group, contraindicating what would be expected if

cholinergic functioning were enhanced in this brain region. This may be because AChE activity responds to ET in an inverted-U shaped dose-response manner which is region specific. Thus, the 1µg/day dose of EDP elicited positive effects on AChE activity in the frontal cortex while this same dose in the hippocampus appeared to exceed the threshold resulting in a negative effect on AChE activity. Given the lack of research in this area and the gross methodological differences between these studies, it is not possible to make inferences at this time.

3.5.4 Effects of Estrogen on Muscarinic and Nicotinic Receptors

Various researchers have found a link between estrogen and muscarinic receptors (Abdalla et al 2000; Abdalla et al 2004; Matucci et al 1996; Munns and Pennefather 1998; Rainbow et al 1980) and nicotinic receptors (Curtis et al 2002; Uki et al 1999) in reproductive tissue. Muscarinic receptors in the hypothalamus and preotic area have similarly been linked to estrogens in relation to reproductive regulatory mechanisms (Dohanich et al 1982; Olsen et al 1988). However, relationships between estrogen and cholinergic receptors have more recently been found in regions of the brain which are unrelated to reproductive functioning. Specifically, ovariectomy has been found to up-regulate muscarinic M_4 receptors (which have a general inhibitory effect on neuronal activity) in the dentate gyrus, hippocampus and frontal cortex of female rats (El-Bakri et al 2002). This was suggested to be in response to a lack of ACh, which is highly probably given the negative effect of ovariectomy on cholinergic markers previously discussed. El-Bakri and colleagues (2002) further speculated that ERs located in cholinergic neurons may have a role in regulating ACh release. Muscarinic M_4 receptor density was later restored to normal after 10 weeks of E_2 treatment, but not progesterone treatment (El-Bakri et al 2002). This may have implications for a protective effect of estrogens on sensorimotor information processing, as blockade of M_4 receptors has since been linked to impaired prepulse inhibition in mice (Ukai et al 2004). Vaucher and colleagues (2002) found decreased M1-like receptor binding in numerous cortical regions and decreased M_2-like receptor binding in the BF, hippocampus, caudate and numerous cortical regions following ovariectomy, in young and old female mice. However, E_2 treatment only restored binding to normal levels in the motor, somatosensory and

perihinal corticies, with no effects observed in the BF, hippocampus or neocortex. This may be due to the length of ET not being long enough (i.e. 3 weeks) to allow significant restoration of muscarinic receptors in these brain regions. However, it should be noted that treatment was initiated at the same time as ovariectomy. Interestingly, Cardoso and colleagues (2004) found ovariectomy to result in a time-dependent increase in muscarinic receptor binding (using [3H]QNB) in the rat hippocampus, when assessed 2, 10 and 15 days post-ovariectomy. Seven days treatment with 10µg/day EB, initiated 15 days post-ovaiectomy, only slightly reversed the effects of ovariectomy on muscarinic receptor binding. Moreover, 3 weeks of EB treatment initiated directly after surgery effectively protected against the effects of ovariectomy, as muscarinic receptor binding was comparable to controls. This provides further support for the 'critical period hypothesis', discussed in Chapter 2.

Other researchers have focused on the effects of estrogens on nicotinic receptors. Morley and colleagues (1983) found that a single dose of estrogen given to prepuberal female mice resulted in an increase in nicotinic receptor binding. Another study used the nicotinic receptor agonist methylcarbamylcholine (MCC) to investigate the effects of chronic E_2 treatment on nicotinic auto-receptor function. It was found that ET caused an elevation in nicotinic receptor density and the subsequent desensitization to MCC in the hippocampus (Lapchak et al 1990), implying a negative effect of chronic ET on cholinergic nicotinic receptor function. This was thought to be related to MCC increasing ACh release. This effect appeared to be region specific as E_2 treatment did not change [^{3}H]MCC binding in the frontal cortex of treated animals relative to controls.

Hosli and Hosli (1999) used combined autoradiographic and immunohistochemical studies to investigate the co-localization of E_2 with cholinergic receptors in explant cultures of rat CNS. The majority of cortical neurons co-expressed ERs (indicated by [^{3}H]-estradiol binding) with muscarinic receptors, whereas hippocampal neurons tended to co-express ERs with nicotinic receptors. Subsequent research revealed that a large proportion of astrocytes in the rat spinal cord co-expressed ERα and ERβ with muscarinic receptors, as well as nicotinic receptors (Hosli et al 2001; Hosli et al

2000). In addition, numerous astrocytes from a primary hippocampal culture also showed muscarinic and nicotinic binding sites to be co-localized with ERs, further suggesting a regulatory role of estrogens on cholinergic functioning. Using the same astrocytes, acute administration of E_2 and muscarine resulted in hyperpolarization of the majority of cells (Hosli et al 2001; Hosli et al 2000). Similar results were found when E_2 and nicotine were co-administered. Collectively, these findings suggest that the co-localization of ERs with cholinergic receptors on neurons and astocytes may have a role in the modulation of neuronal functions that require cholinergic activation. More work into the specific interaction of nicotinic and muscarinic receptors with ET is needed.

3.5.5 Lesion Studies

In addition to the reported direct modulatory role of estrogens on markers of cholinergic function, evidence also exists for E_2's neuroprotective effects on the cholinergic system as demonstrated by lesions studies. Rabbani and colleagues (1997) found that a single dose of E_2 administered 1 day prior to partial fimbria-fornix lesioning largely prevented the usual decline in ChAT-IR in the medial septum of OVX rats (4% loss compared to 44% loss). Furthermore, chronic E_2 treatment for 5 days prior to lesioning also significantly attenuated the loss of ChAT-IR. Conversely, Bora and colleagues (2005) failed to find any difference in BF ChAT-IR between E_2-treated and vehicle-treated rats with complete fimbria-fornix transections, despite a significant protective effect of E_2 on ChAT mRNA levels. Findings may differ to those of Rabbani and colleagues (1997) given that the latter study used complete fimbria-fornix transections, and treatment was delayed (by 3 months) post-ovariectomy. However, ChAT mRNA was significantly protected by E_2 treatment, suggesting that E_2 may be able to affect ChAT neurochemical phenotype more readily than immunoreactivity, which may be dependent on the dosage or duration of treatment.

Using excitotoxic NMDA-induced lesions of the NBM, Horvath and colleagues (2002) found that 2 weeks EB treatment had no protective effect on ChAT-IR, VAChT-IR or AChE fibre density, even though ET had significantly increased these

cholinergic makers prior to lesioning. Another study investigating NMDA olfactory bulb lesions found that one week of pre-treatment with E_2 attenuated the lesion-associated loss of choline uptake in the cingulate cortex, a region associated with attention and learning (Sohrabji et al 2000). In contrast, Galani and collegues (2002) found that one week of ET prior to SAP lesioning did not have an effect on the extent of cholinergic neuronal damage in a sample of gonadally-intact male rats, nor were there any sex differences in cholinergic damage between male and female rats (Galani et al 2002). This may have been due to the selectivity of SAP for cholinergic neurons, as discussed earlier in this chapter. Unfortunately, Galani and collegues (2002) did not examine the effects of ET in a sample of female rats injected with SAP. Gibbs (2002) however, did administer SAP to OVX rats treated with 4 weeks of E_2 or E_2 plus progesterone and found it did not protect against the SAP-induced decline in BF ChAT activity and ChAT-IR. Similar results have been observed in the MS/dbB and CA1 region of the hippocampus (Lam and Leranth 2003). In addition Gibbs (2002; 2007) found that E_2 treatment did not protect against the SAP-induced impairment in acquisition of a spatial DMP task. Despite this, a significant correlation between DMP acquisition and ChAT activity in the frontal cortex and hippocampus was found (Gibbs 2002), again supporting a possible regulatory role of E_2 on cholinergic function in key brain regions essential for higher-order cognitive processes. Findings are somewhat mixed but are most likely due to methodological inconsistencies, such as mode of estrogen and SAP administration, length of treatment and dosages used (for more information see section 3.2). Nevertheless, overall findings in this area of research are positive and provide strong support for E_2 as a neuroprotective agent within the cholinergic system. The mechanisms by which cholinergic functioning deteriorates after the loss of endogenous estrogens and the restoration of cholinergic markers with ET, have yet to be fully characterised and warrants further investigation.

3.6 Estrogen Treatment, Cognition and Cholinergic Manipulation

3.6.1 Animal Studies

The scopolamine model, as discussed earlier, has frequently been utilized to test the effects of E_2 treatment on cholinergic functioning and subsequent cognitive performance in animals. Dohanich and colleagues (1994) were the first to find that three days of high dose E_2 benzoate (25g) alone or in combination with progesterone, significantly protected against the impairing effects of scopolamine on a T-maze working memory task in female rats. A later study by Packard and Teather (1997) examined memory retention using a water maze task. Rats in the active treatment groups were concurrently administered acute post-training injections of E_2 (0.2mg/kg) plus either a subeffective dose of scopolamine (0.1 mg/kg) or saline, while the placebo (saline) treatment groups were concurrently administered either the peripheral muscarinic antagonist methylscopolamine (0.1 mg/kg) or saline. There were no significant differences in memory performance between the E_2 /scopolamine group and either of the two control groups. The authors concluded that this low dose of scopolamine blocked the cognitive enhancing effects of E_2, given that the E_2/saline-treated rats demonstrated significantly better memory retention than placebo/saline-treated rats. If the dose of scopolamine had been large enough to elicit an effect when given alone it would be assumed that estrogen protected against the damaging effects of scopolamine, however because a 'subeffective' dose was used the results imply that low dose scopolamine can suppress the possible cognitive enhancing effect of E_2 treatment. This may be due to E_2 and scopolamine being administered concurrently, suggesting that E_2 treatment may act by building up a defense for cholinergic neurons over a period of time. Furthermore, findings were not likely due to blockade of peripheral muscarinic receptors (and therefore possible effects on swim speed), as rats administered E_2 plus the muscarinic receptor antagonist methylscopolamine (which acts on the parasympathetic nervous system) performed significantly better than controls. In addition Packard and Teather (1997) also found that a subeffective dose of E_2 (0.1mg/kg) combined with a subeffective dose (0.1mg/kg) of oxotremerine, a muscarinic receptor agonist, resulted in enhanced

memory performance, suggesting that these two substances worked synergistically to enhance cholinergic functioning.

Following on from this early research a number of other studies have found both short and long-term E_2 treatment (ranging from 3 days-12 months) to significantly attenuate the scopolamine-induced impairments on measures of learning (Fader et al 1998; Gibbs et al 1998), working memory (Fader et al 1999; Gibbs 1999; Tanabe et al 2004) and memory retention (Tanabe et al 2004) using various tasks such as passive avoidance, delayed match to position, Morris water maze, RAM and T-maze tasks. Gibbs (1999) found that impairments in working memory were significantly attenuated by E_2 treatment when scopolamine was administered via intrahippocampal but not systemic injection. Conversely, Fader and colleagues (1998) found that E_2 treatment had a protective effect on T-maze acquisition regardless of whether scopolamine had been administered hippocampally or systemically, which may be due to the systemic dosage of scopolamine being much lower than that used in the former study (0.2mg/kg vs. 1mg/kg). Despite these discrepancies, findings suggest that E_2 protects hippocampal-mediated cognitive functions.

While the majority of research in this area has found positive results, one research group failed to find any protective effect of short-term E_2 treatment against scopolamine on working and reference memory in aged female mice (Markowska and Savonenko 2002). However, Savonenko and Markowska (2003) later found that E_2's ability to enhance cholinergic functioning diminished with age, given that middle-aged female rats (12-13 months old) treated with E_2 showed no impairment after scopolamine while E_2-treated aged female rats (20 months old) did demonstrate learning deficits. This implies that, in regards to E_2 as a possible treatment for AD and neurological disorders alike, E_2 treatment may not be effective if administered after initial onset of the disorder but may protect against cholinergic deterioration if administered prior to marked cognitive decline.

To date, only two studies have investigated the protective effects of estrogens following cholinergic manipulation using a primate non-human sample. Voytko (2000) found that there was no change in performance of a delayed response task

following a scopolamine challenge, when animals had been OVX (and thus deprived of endogenous estrogens) for 2 months as compared to pre-operatively. In a later study Voytko (2002) administered 0.01-0.04 mg/kg of scopolamine to a sample of female monkeys 4 months after chronic E_2 or placebo treatment. As mentioned in Chapter 2, spatial attention (release time) improved significantly from baseline for the E_2 group, however after the scopolamine challenge performance on this task worsened. The control group however, tended to have faster release times (hence better visuospatial attention) post-scopolamine, leading Voytko (2002) to postulate that the decline in estrogen post-ovariectomy may have caused heightened sensitivity of cholinergic neurons and that only extreme muscarinic receptor blockade would elicit a cognitive deficit. However, this does not explain why the control group was not impaired in performance post-scopolamine. Voytko (2002) concluded that E_2 did not protect against scopolamine, which impaired general alertness and disrupted the disengagement process of attention.

Using a different model of cholinergic impairment Daniel and Dohanich (2001) found the muscarinic M_2 receptor antagonist BIBN 99 blocked the cognitive enhancing effect of short-term (3 days) E_2 treatment on working memory performance, assessed using a RAM task. More recently, Daniel and colleagues (2005) used the M_2 receptor antagonist AFDX 116 to specifically investigate the role of hippocampal M_2 receptors in estrogen-mediated working memory. They used bilateral cannulae implants in conjunction with osmotic minipumps to deliver AFDX 116 directly into the hippocampus or cortex (control site). The results showed AFDX 116 delivered to the hippocampus significantly suppressed the enhancing effects of 3 days EB treatment on performance of the water maze task, however there was no dampening of EB effects when AFDX 116 was delivered to the control site, suggesting that cholinergic muscarinic receptors in the hippocampus specifically are required for E_2-modulated improvements in working memory. This finding is novel given the site-specific effects of E_2 and calls for further research of this kind to investigate a wider range of cognitive processes, not only in reference to the hippocampus but key cortical areas as well, namely the PFC.

3.6.2 Human Studies

To date, only one research group has investigated the protective effects of E_2 following a pharmacological cholinergic challenge in humans. Dumas and colleagues (2006) used scopolamine and mecamylamine (nicotinic agonist) to test the neurocognitive effects of three months treatment with 1mg/day oral E_2 on the cholinergic muscarinic and nicotinic receptor systems respectively, under a double-blind placebo-controlled cross-over design in 15 post-menopausal women. Each participant underwent 5 different challenges; high (5µg/kg) and low (2.5µg/kg) dose scopolamine, high (20mg) and low (10mg) dose mecamylamine and placebo. Scopolamine successfully impaired cognitive performance on all measures in the domains; attention, verbal memory and non-verbal memory (other than the Benton Visual Retention Test), while mecamylamine only impaired performance on some of the attention measures and the non-verbal memory measures. Dumas and colleagues (2006) found E_2 treatment had a significant attenuating effect on the scopolamine-induced impairment on the CFF task and RT on the continuous performance task. Similarly, E_2 treatment also attenuated the impairing effect of mecamylamine on RT of the CRT task and RT on the continuous performance task. These effects were not likely a result of direct interactions with scopolamine or mecamylamine at the receptor site as there were no E_2 treatment effects on objective or subjective measures of sedation. However, E_2 had no protective effects on accuracy measures of the above tasks or any of the other measures in the cognitive battery including the DSST, divided attention test, the Buschke Selective Reminding Task (BSRT), Verbal Paired Associates, paragraph recall, the Repeated Acquisition Task and the BVRT, suggesting E_2 may have selective protective effects on certain cognitive domains, namely attention and speed of information processing/psychomotor function. Thus, one might speculate that E_2 acts specifically on corticopetal cholinergic neurones and the effects of E_2's actions become behaviourally apparent only when cholinergic tone is compromised, given that no cognitive effects were found between placebo and E_2 after the three months of treatment.

A later study by Dumas and colleagues (2008) utilised a very similar protocol, however post-menopausal women were divided into two groups based on their age

(ie. younger = age 50-62 and older = age 70-81). In addition, and in contrast to their earlier study, participants were administered 1mg/day oral E_2 for one month and then increased to 2mg/day for the remaining 2 months, and only the 2.5µg/kg dose of scopolamine and the 20mg dose of mecamylamine were employed. Interestingly, E_2 treatment significantly attenuated the scopolamine-induced deficit in verbal memory (BSRT) for the younger group of post-menopausal women while older group showed greater impairment with the E_2 treatment compared to placebo treatment following scopolamine. This finding is novel as the BSRT was unaffected in the earlier study, yet the latter study conversely failed to find significant effects on the tasks of attention. The discrepancies between the two studies may be due to a combination of age specificity as well as the increase in E_2 dosage. However, it should be noted that when the two groups were analysed separately scopolamine successfully impaired all three performance measures of the BSRT (total recall, recall consistency, recall failure) for the younger group while only the total recall measure was significantly impaired by scopolamine in the older group of participants. Furthermore, scopolamine was more frequently successful at impairing the performance measures of the attention tasks (CFF, CRT, continuous performance task) for the younger group as opposed to the older group, thus there were less instances where E_2-treatment effects on cholinergic indicies could be detected for the latter group of women. In line with the earlier study, mecamylamine was again less efficient at impairing performance on the majority of tasks used and there were no significant E_2-treatment effects under the placebo challenge condition. Despite this, as mentioned above, there were significant interactions of treatment group by age for BSRT under the scopolamine condition. These findings support the critical period hypothesis (see chapter 2 for more information), and suggest that E_2's protective effects after the menopause may be via interactions with cholinergic muscarinic receptor mechanisms.

Imaging studies, although just as scarce as challenge studies, have also provided evidence for estrogenic effects on the cholinergic system in humans. Smith and colleagues (2001) used SPECT and the radiotracer [^{123}I]iodobenzovesamicol ([^{123}I]IBVM) to compare the VAChT binding index between healthy postmenopausal women who were HT-users and those who were not. VAChTs have the role of

loading acetylcholine into secretory organelles which consequently makes acetylcholine available for secretion by the pre-synaptic neuron, thus VAChT binding is an indicator of cholinergic synaptic terminal density. Worth noting, this study was relatively well-controlled given the nature of the study as HT was reportedly uninterrupted since initiation of treatment after the menopause and all 16 of the HT-users had been taking 0.625mg/day Premarin (the most widely prescribed CEE), although half of these participants were also taking a progestagen (medroxyprogesterone acetate). Furthermore there were no differences in age, education level, age at menopause or the number of years postmenopausal, between the two groups (some variables were matched). Despite this, Smith and colleagues (2001) were unable to find any significant difference in VAChT binding between HT-users and non-users, however they did find that women who used ET (*N*= 8) had significantly (*M*= 25%) higher VAChT binding in the posterior cingulate than combined EPT-users (*N*= 8), further supporting the theory that the addition of a progesterone may mitigate the positive effects of ET. In addition significant positive correlations between length of HT-use (which appeared to range from 5 to 25+ years) and binding indexes in the frontal, parietal, and temporal corticies as well as the anterior and posterior cingulate were found. There were no significant correlations for sub-cortical regions, suggesting long-term HT may selectively protect cortical cholinergic functioning.

More recently, Norbury and colleagues (2007) investigated the relationship between muscarinic receptor density, long-term ET and cognition using SPECT and the novel radioligand (*R,R*) [^{123}I]-I-QNB (which binds predominantly to M1/M4 muscarinic receptors). The sample comprised postmenopausal ET-users and never-users (*M* age = 65) as well as young pre-menopausal women (*M* age = 29) not on the contraceptive pill. Young women (when scanned during the high-estrogen phase of the menstrual cycle) had significantly higher muscarinic receptor binding than either group of post-menopausal women in numerous brain regions, as expected. Muscarinic binding was also found to be significantly higher in the striatum, hippocampus, lateral frontal cortex and thalamus of postmenopausal ET-users when compared to never-users. Although only one cognitive measure was administered besides the WASI (used as an indicator of overall IQ), the ET-users were found to make fewer perseverative

errors on the CVLT in comparison to the never-users, suggesting long-term ET (on average 18 years) has positive effects on executive functioning. However, Norbury and colleagues (2007) failed to find any correlations between binding potential and performance measures of this task in postmenopausal women. Interestingly, for ET-users, plasma E_2 levels were positively correlated with binding potential in the left hippocampus and temporal cortex, suggesting that circulating plasma hormone levels may reflect hormone levels in the brain. However, this is speculative as the level of hormones present in these brain regions was not known and may have differed to plasma levels [given the brain can produce its own estrogens locally (Simpson 2003)]. Lastly, although Smith and colleagues (2001) found that the duration of HT corresponded to a marker of cholinergic functioning, there was no significant relationship between (*R*,*R*) [^{123}I]-I-QNB binding and duration of E_2 treatment in this study.

Despite the limited number of human studies in this area of research there is ample evidence from molecular and biochemical animal research to support significant neuroprotective effects of E_2-treatment on the cholinergic system. In line with the behavioural literature reviewed in Chapter 2, the pattern of ET-effects on cholinergic markers suggests there may be a possible dose-by-region specific effect, where the optimal level of estrogen within the hippocampus may differ to that of the PFC and other brain regions**.** In addition, the behavioural animal literature further implies an important role for the cholinergic system in mediating E_2-treatment effects on cognition, in particular learning and spatial working memory. Thus, there are clear grounds for further exploration of the estrogen-cholinergic hypothesis in human populations, which has implications for not only brain-aging but the treatment of neurodegenerative and neurological disorders characterised by cognitive deficits, of interest schizophrenia.

Chapter 4
Rationale and Thesis Objectives

4.1 Rationale & Aims

The general aim of this thesis was to extend upon the understanding of the effects of E_2 treatment on cognitive functioning in women. Over the past decade there has been an exponential growth in interest regarding estrogens and their effect on cognition, with a wealth of biochemical and molecular evidence to support E_2 in particular, as a neuroprotective agent in the brain. Estradiol's protective mechanisms have primarily occurred in brain regions essential for cognitive functioning, namely the hippocampus and frontal cortex. In addition, the vast body of epidemiological, observational and experimental research in post-menopausal women further suggests E_2 treatment has protective and potential cognitive-enhancing effects. However, the mechanisms underlying E_2's actions in the brain are largely unknown. Although E_2 has been linked to numerous neurochemicals within the brain, the most compelling evidence comes from E_2's interactions with the cholinergic system, a neurotransmitter system with a well-established fundamental role in learning, memory consolidation, declarative and spatial memory, working memory and attentional and information processing.

Experiment One of this thesis therefore, aimed to investigate whether E_2's effect on cognitive function in humans is mediated via the cholinergic system. More specifically Experiment 1 aimed to examine whether short-term E_2 treatment would protect against the cognitive impairing effects of the muscarinic receptor antagonist scopolamine, in a sample of healthy young women of child-bearing age.

Estradiol has been proposed as a possible treatment for cognitive deficits inherent to a number of neurological and mental disorders, including schizophrenia, based on the reported benefits of E_2 treatment on cognitive function in postmenopausal women. Since the recent acknowledgement of cognitive impairment as an independent and unequivocal feature of schizophrenia, the need for effective treatment in alleviating cognitive deficits has become much sought after. These impairments in cognition have been suggested to arise from abnormalities in the integrity and regulation of the basal forebrain cholinergic system and projection pathways, based on the essential role of this neurotransmitter system in numerous cognitive processes, as well as the

compromised functioning of the cholinergic system (including muscarinic receptor dysfunction) observed in patients with schizophrenia.

Experiment Two of this thesis therefore, aimed to investigate the cognitive effects of adjunctive E_2 treatment in women of child-bearing age with schizophrenia.

The effects of E_2 treatment on cognitive function in healthy controls (Experiment One) and patients with schizophrenia (Experiment Two) was examined in women of child bearing age. There were a number of reasons for choosing this cohort, namely; (1) very little research into the effects of E_2 treatment in young women exists, thus it is simply not known whether short-term E_2 treatment given to women with normal menstrual cycles would alter cognitive performance, (2) the vast biochemical and molecular evidence suggests that E_2 treatment may have cognitive enhancing effects regardless of age, and (3) illness onset for women with schizophrenia is typically around the age of 27-30 (Hafner 2003; Salokangas et al. 2003).

Chapter 5

Experiment One: Estradiol Treatment & its Interaction with the Cholinergic System: Effects on Cognitive Function in Healthy Young Women

5.1 Introduction

The steroid hormone E_2 is widely recognized as a multifaceted neuroprotective agent in the brain and CNS, with significant protective effects on certain cognitive processes, as described in Chapters 1 and 2. In summary, epidemiological studies have shown postmenopausal women taking ET or HT performed better than non-users on tasks assessing the following cognitive processes; verbal fluency (Grodstein et al 2000; Miller et al 2002; Szklo et al 1996), verbal and visual memory (Henderson et al 1996; Jacobs et al 1998; Kampen and Sherwin 1994; Maki et al 2001; Resnick et al 1998; Resnick et al 1997; Sherwin 1988; Smith et al 2001a; Stephens et al 2006; Verghese et al 2000), working memory (Duff and Hampson 2000; Keenan et al 2001; Miller et al 2002) attention (Friebely et al 2001; Smith et al 2001a), abstract reasoning (Jacobs et al 1998; Keenan et al 2001; Schmidt et al 1996), information processing (Hogervorst et al 1999; Kritz-Silverstein and Barrett-Connor 2002) and global cognitive functioning (Kimura 1995; Rice et al 2000; Steffens et al 1999). Similarly, experimental studies have reported cognitive enhancement with various doses of ET/EPT, administered via various different routes (Asthana et al 2001; Duka et al 2000; Joffe et al 2006; Krug et al 2006; Linzmayer et al 2001; Phillips and Sherwin 1992a; Saletu 2003; Shaywitz et al 2003). However, estrogen's ability to preserve or enhance cognitive function continues to be debated as a number of studies have reported negative or no cognitive-enhancing effects of HT in postmenopausal women (Alhola et al 2006; Almeida et al 2006; Barrett-Connor and Kritz-Silverstein 1993; Espeland et al 2006; Janowsky et al 2000; Kang et al 2004; Leblanc et al 2007; Morse and Rice 2005; Polo-Kantola et al 1998; Yaffe et al 2006). Inconsistencies in the literature are most likely due to a lack of controlling for confounding variables such as age, education, general health, socioeconomic status, type, dosage and duration of treatment, type of menopause (surgical/natural), climacetric symptoms and not correcting for multiple comparisons/analyses. Comprehensive review and meta-analysis has lead to the general conclusion that HT has a small but significant positive effect on measures of verbal memory, abstract reasoning and information processing (Hogervorst et al 2000; Sherwin 2006; Zec and Trivedi 2002). Interestingly, there are no studies that

have investigated the effects of E_2 treatment in healthy young or pre-menopausal women.

More recently research has centered around the molecular mechanisms underlying E_2's neuroprotective effects, which have implications not only for improving cognitive function AD and older age but also in schizophrenia (for reviews see Cyr et al 2002; Garcia-Segura et al 2001; Halbreich and Kahn 2003; Osterlund and Hurd 2001). Biochemical evidence suggestive of neuroprotective actions of estrogens (predominantly E_2) include; protection from oxidative stress (Behl et al 2000; Behl et al 1997; Behl et al 1995; Mize et al 2003; Numakawa et al 2007), facilitation of neurite outgrowth and proliferation (Brinton et al 1997; Cambiasso and Carrer 2001; Carrer et al 2005; Fester et al 2006; Murphy et al 1998; von Schassen et al 2006; Zhou et al 2005), protection from β-amyloid toxicity (Fitzpatrick et al 2002; Goodman et al 1996; Keller et al 1997; Zhang et al 2001) and modulation of various neurotransmitter systems, including the dopaminergic (D'Astous et al 2005; Dazzi et al 2007; Dluzen 2000; Jourdain et al 2005; Landry et al 2002; Le Saux and Di Paolo 2006; Lee and Mouradian 1999; Liu and Dluzen 2006), serotonergic (Birzniece et al 2001; Kugaya et al 2003; Osterlund et al 1999; Sumner and Fink 1995) and cholinergic systems (for review see Chapter 3).

Interaction of estrogens with the cholinergic system in particular, has been suggested to partially explain estrogenic effects on cognitive function (Gibbs 2000c; Tinkler and Voytko 2005; Toran-Allerand et al 1992). This proposed mechanism is highly feasible given cholinergic input to fronto-limbic and fronto-striatal regions (Selden et al 1998), and the abundance of evidence supporting this system's prominent role in modulating fundamental cognitive processes, particularly attention, learning, declarative memory and working memory (Ellis et al 2006; Everitt and Robbins 1997; Sarter et al 2005). As outlined in Chapter 3, this hypothesis is supported by animal studies which show; (1) estrogen receptors are co-localized on cholinergic neurons (Miettinen et al 2002; Shughrue et al 2000; Toran-Allerand et al 1992), (2) choline acetyltransferase activity is increased in cholinergic basal forebrain neurons after E_2 treatment (Gibbs et al 1994; Luine 1985; McMillan et al 1996), (3) high-affinity choline uptake is increased in the frontal cortex and hippocampus after E_2

treatment (Gibbs 2000a; Singh et al 1994), (4) E_2 treatment can enhance working memory via interaction with muscarinic (M_2) receptors in the hippocampus (Daniel et al 2005) and (5) E_2 treatment protects against the cognitive-impairing effects of the muscarinic receptor antagonist scopolamine, on passive avoidance, delayed match-to-position and T-maze tasks, measures of learning, declarative memory, and working memory in animals (Dohanich et al 1994; Fader et al 1998; Fader et al 1999; Gibbs 1999; Gibbs et al 1998; Savonenko and Markowska 2003; Tanabe et al 2004).

The interaction between estrogens and the cholinergic system in humans is less established. Smith and colleagues (2001b) found that length of HT in postmenopausal women was positively correlated with VAChT binding indexes in certain brain regions including the frontal and cingulate cortices, areas essential for attention, learning and memory. However, they were unable to find a difference in VAChT binding between HT users and non-users (Smith et al 2001b). Using SPECT and the muscarinic ligand (*R,R*) [^{123}I]-I-QNB, Norbury and colleagues (2007) recently found higher muscarinic receptor density in the striatium, hippocampus, frontal cortex and thalamus of ET users compared to non-users. To date only one research group has investigated the interaction between E_2 treatment and the cholinergic system with regard to neurocognitive effects in an experimental design. Dumas and colleagues (2006) found three months of 1 mg/day oral E_2 treatment in postmenopausal women significantly attenuated scopolamine-induced deficits on measures of attention and reaction time. In addition ET also attenuated deficits caused by mecamylamine (a nicotinic receptor antagonist) on two measures of reaction time. However, no interaction between E_2 treatment and the cholinergic system were found on measures on verbal or visual learning and memory, or any of the accuracy/error measures of the attention tasks. More recently Dumas and colleagues (2008) reported a protective effect of E_2 treatment on verbal episodic memory in younger (aged 50-62) compared to older (aged 70-81) post-menopausal women following a scopolamine challenge, suggesting the possibility of a critical period for ET's beneficial effects on the cholinergic system for women after the menopause (see Chapter 3 for more information).

The neurocognitive effects of ET and its interaction with the cholinergic system has not been investigated in healthy young women of child-bearing age or following a shorter duration of ET. Before any real conclusions can be made it is important to test the relationship between estrogen, the cholinergic system and cognition in healthy young women, to determine whether estrogen's effects are limited to the postmenopausal age demographic. Given that E_2 has been suggested to be a possible treatment for the alleviation of cognitive deficits in not only neurodegenerative disorders but also neurological disorders such as schizophrenia (Hoff et al 2001; Kulkarni et al 2001), it is necessary to test the effects of estrogen treatment in not only young women with schizophrenia (as we have done in Experiment 2 of this thesis, see Chapter 6) but also young healthy women not taking any medication, in order to truly gage an understanding of the neurocognitive effects of estrogen treatment in this age cohort. Therefore, the current study examined; (1) the effects of one month of 100µg/day E_2 treatment on cognitive function in a sample of healthy young women and (2) the interaction between E_2 treatment and the cholinergic system, specifically whether one month of E_2 can attenuate the scopolamine induced deficits in cognitive function for this age group.

5.2 Methods

5.2.1 Participants

34 healthy female volunteers (Mean age = 22.59 ± 4.45) aged between 18 and 38 years (Mean weight = 57.87 ± 12.57) were recruited for this study from universities around Melbourne. All participants underwent a comprehensive medical screening by a physician who assessed the individual's physical and mental health (see Appendix C and D for participant screening questionnaire and physical examination criteria respectively). Women were excluded if they were pregnant, lactating, peri- or post-menopausal, had a current or past psychiatric illness, epilepsy or a familial history of epilepsy, peptic ulcers, diabetes or a history of diabetes, malignancies or history of familial breast cancer or fibrocystic mastopathy, hyperlipidemia, neurologic disease or previous head injury, cardiovascular or endocrine abnormalities, impaired liver or kidney function or had a history of drug or alcohol

abuse. Women were also excluded if they were taking medication (including herbal remedies and vitamins), if they were smokers, had irregular menstrual cycles or had been on the pill in the last month. All volunteers gave their written informed consent to take part in the study which was approved by the Swinburne Human Research and Ethics Committee (see Appendix E for plain language statement and consent form).

5.2.2 Procedure

Participants were randomized to receive either 100µg/day transdermal E_2 (Dermestril or Estraderm100; Faulding Pharmaceuticals/Mayne Pharma [USA] Inc.) or transdermal placebo under double-blind conditions (supplied by Faulding Pharmaceuticals/Mayne Pharma [USA] Inc.) (see Appendix F for group/drug randomization). Estradiol treatment was in the form of 8mg adhesive skin patches (9 in total) which were changed every 3-4 days resulting in a continuous release of E_2 throughout the duration of the study. Transdermal administration allows E_2 to directly enter the bloodstream without going through first-pass metabolism by the liver, thus the 100µg/day dosage is expected to elevate circulating plasma E_2 levels by approximately 275pmol/L (i.e. 75pg/ml) (Mercuro et al 1997). Previous research has found this mode and dosage of treatment to significantly enhance cognition in postmenopausal women (Duka et al 2000; Krug et al 2006; Krug et al 2003). Prior to baseline testing participants underwent repetitive training on computerized tasks in order to minimize practice effects (McClelland 1987; Wesnes and Pincock 2002).

After completion of four practice sessions a start date was estimated based on anticipation of the participant's first day of menstruation, as all participants were required to begin the study during the early-mid follicular phase (days 1-10) to control for endogenous estrogen levels. Scheduled start dates were readjusted when needed to account for this requirement. On testing days subjects were instructed to have a standard breakfast (toast and juice recommended) prior to attending the Brain Sciences Institute (BSI), with emphasis that the same type of breakfast must be consumed on the morning of each test session. Participants were also informed that they were not to consume any caffeine or alcohol 24h prior to testing. For the first test session participants arrived at 0830h and performed the following questionnaires

and cognitive tests: modified version of the Menstrual Cycle Questionnaire (excluded item 'h', see Appendix G), National Adult Reading Test (NART), Rey Auditory Verbal Learning Test (RAVLT), Controlled Oral Word Association Test (COWAT), Spatial N-back Task, Digit Vigilance Task, Stroop Color and Word Test, Simple and Choice Reaction Time tasks and the Visual Analogue Mood Scale (VAMS). Upon completion of baseline testing, participants received the E_2 or placebo patches to take home and were given detailed instructions on how to apply them. Participants were provided with detailed information on how to apply the patches (see Appendix H), and were instructed to continue wearing the patches until the end of the third test session. Participants were monitored for compliance and possible adverse effects via weekly phone calls. During each phone call it was stressed that a patch had to be worn at all times. In the instances where a patch did not adhere to the skin, it was replaced immediately and the researcher subsequently provided the participant with an extra patch. Participants were also required to retain the wrappings from each patch and present them at the end of the one month period in order to enforce compliance.

Participants returned to the BSI for test session two on day 28 and completed the same cognitive tasks as stated above (excluding the NART and with the addition of the Critical Flicker Fusion Task). Participants were then given a drug challenge of either scopolamine (0.4mg/1ml solution) or placebo (1ml saline) in the form of an intramuscular injection by a trained registered nurse. This dosage has been previously found to induce significant cognitive impairment in healthy subjects (Ellis et al 2006; Little et al 1998; Robbins et al 1997). Previous research has established that the central pharmacodynamic effects of scopolamine peak between 60 and 180min (Ebert and Kirch 1998; Safer and Allen 1971), thus cognitive testing (duration 1.5h) was resumed after a 90min break. The same cognitive tests were repeated except for the COWAT (only administered pre and post E_2/placebo treatment). After a washout period of two days (based on the scopolamine elimination half life of 2.4 ± 1.4h, and a minimum of 5 half lives washout period), participants returned for the third test session and repeated the procedure carried out in session two, however the opposite drug (scopolamine/placebo) was administered (see Figure 7 for schematic timeline of testing regimen).

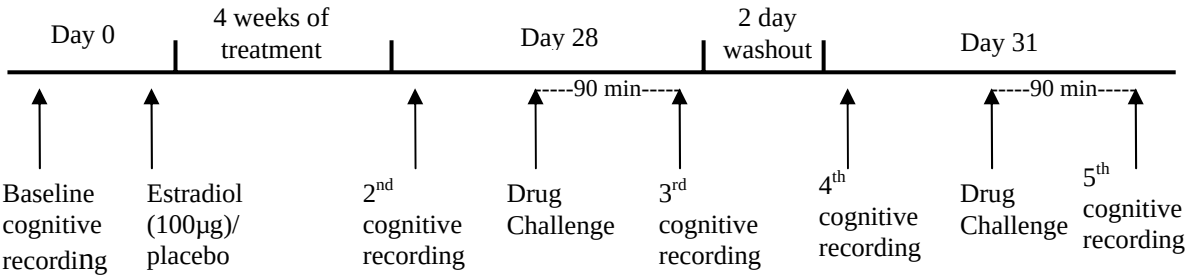

Figure 7. Timeline of Testing Schedule

During the 90min break subjects were allowed to read, study or watch videos. During this time and during the post-drug cognitive testing, blood pressure and pulse rate were monitored every 30 min. The Adverse Symptoms Checklist was also administered pre and post scopolamine/placebo injections. The reason for including a baseline session on each of the drug challenge days was to: 1) eliminate any variation in performance due to unforeseen events on the day of testing and 2) remain consistent with the cognitive demands on both days 28 and day 31 (as day 28 required pre-injection cognitive testing in order to investigate hypothesis 1).

5.2.3 Cognitive Battery

The cognitive tasks (and specific cognitive domains) were chosen based on their sensitivity to ET from postmenopausal studies while also taking into consideration cognitive processes commonly impaired in schizophrenia. This included tasks that assess the following cognitive domains; declarative verbal memory and learning, verbal fluency, working memory, attention, cognitive flexibility and psychomotor function and information processing. Neuropsychological tests included in this battery consist of both renowned paper and pencil tests and validated computerized task from the Cognitive Drug Research (CDR) Computerized Assessment System (CDR Ltd. Goring-on-Thames: UK) and in-house tasks developed at the Brain Sciences Institute. All cognitive measures had good to excellent test-retest reliability (coefficients range from .66-.97; for more detail see Lezak et al 2004). All computerized tasks had alternate versions for each test session.

5.2.3.1 Declarative Verbal Memory and Learning

Rey Auditory Verbal Learning Test (RAVLT): The RAVLT (Rey 1964; Taylor 1959) measures ones ability to encode, consolidate, store and retrieve verbal information from memory (Schmidt 1996). This test involved a list of 15 monosyllabic words (list A) which were read aloud to the participant who were then instructed to recall as many words as possible immediately after presentation. This procedure was repeated a total of 5 times (trials 1-5). After a 30 min delay participants were asked to recall freely as many words from list A as possible. The outcome measures used in the current study were the total words recalled for list A (trials 1-5) and the long-delay free-recall trial, which are recognized as measures of overall declarative verbal learning and memory and long-term memory respectively (Lezak et al 2004). Two parallel versions (see Lezak et al. 2004) were employed in addition to the original so that participants did not repeat the same version of the task in succession.

5.2.3.2 Verbal Fluency

Controlled Oral Word Association Test (COWAT): The COWAT is a measure of verbal fluency, which can be described as one's ability to generate words related to a given category in a limited amount of time (Benton and Hamsher 1976). Participants were asked to verbally produce as many words that began with a particular letter (e.g. F), in 1min. This was repeated using the letters A and S. Participants were also instructed to try not to produce proper nouns, numbers, repeat words, or repeat the same word with a different suffix. The outcome measure for verbal fluency was the total number of correct words produced across the three trials.

5.2.3.3 Working Memory

Spatial Working Memory N-Back Task: This task was developed at the BSI and run using the Pipscript software program (Pipingas, 1999, Brain Sciences Institute, Victoria, Australia) and has previously been found to be sensitive to cholinergic manipulation (Green et al 2005). The task involved 80 successive presentations of a single white dot in various locations on the computer screen; each appearing for 500ms. Presentation of a dot was followed by a 3000ms delay, during which time a fixation cross was displayed. Participants were instructed to fixate their gaze on the cross until the next dot appeared. This n-back task consisted of two levels, the 1-

back and the 2-back, which were run independently of each other. During the 1-back task participants were asked to answer 'yes' or 'no' (using a button box) as to whether the location of each dot was in the same location as the dot presented directly before it. Participants were told to respond to every dot, except for the first one, as quickly as possible and that both reaction time and accuracy were being recorded. The 2-back task was similar, however participants were asked to determine whether the location of a dot on the screen was in the same location as the dot presented two before it (see Appendix I for instructions presented to participants). The accurate execution of this task requires the ability to constantly update and manipulate information that is stored in working memory. The outcome measures for these two tasks were the percentage of correct responses and the mean reaction times.

5.2.3.4 Attention

Digit Vigilance (CDR): This task is a measure of sustained attention and lasts for 3 min. A target digit was randomly chosen and displayed on the right hand side of the computer screen for the entire duration of the task. A continuous series of numbers were then presented in the middle of the screen at the rate of 150/min. The participant was instructed to press the 'yes' button as quickly as possible whenever they saw the target number appear in the middle of the screen. The target digit randomly appeared in the series of continuous digits a total of 45 times. The outcome measures used in the present study were the percentage of targets correctly detected and the mean reaction time for detecting those targets.

5.2.3.5 Cognitive Flexibility

Stroop Color and Word Test: The traditional paper and pencil version of the Stroop Color and Word Test (Golden 1978; Stroop 1935) was administered and is a measure of executive function, response inhibition and attentional control, specifically requiring the ability to flexibly adjust attention (MacLeod 1991). This task had three separate trials, each lasting 45sec. Participants were instructed to read aloud, as fast as possible, a series of; names of colors (green, blue, red) written in black ink, blocks of actual colors (appearing as 'XXXX') and colors written as the word of an incongruent color (such as the word 'green' written in red ink). This third trial tests

the speed of recognition and response when two perceptual processes conflict. Because individuals have a learned automatic response to read words rather than the colors they are written in, a conflict arises when the innate response must be inhibited and a new set of rules applied, thus an interference occurs. The outcome measure used in the current study was the interference score, calculated using the total number of words read (within the 45sec) for each of the three trials.

5.2.3.6 Psychomotor Function and Information Processing

Simple Reaction Time (SRT) Task (CDR): This task is a measure of psychomotor speed. The SRT task measures how quickly one can respond to a visual stimulus with a motor response. Participants were instructed to press the 'yes' button as quickly as possible, every time the word 'yes' appeared in the middle of the screen. The stimulus appeared after varying delay intervals and remained on the screen until the response was made. Reaction time was measured in milliseconds.

Choice Reaction Time (CRT) Task (CDR): This task is a measure of information processing and psychomotor speed. It is similar to the SRT task, however there are two different responses that the participant must choose between. Either the word 'yes' or the word 'no' was displayed on the screen and participants were instructed to press the corresponding button as quickly as possible. Similar to the SRT task there were varying inter-stimulus delay intervals and reaction time was measured in milliseconds.

Critical Flicker Fusion (CFF) Test (CDR: The CFF task was used in the current study as a measure of information processing, alertness and drug-induced sedation. Participants held the cylindrical light box, and rested their preferred eye on the brim of the tube. They were instructed to fix their gaze on the two flickering lights at the base of the tube which either flickered at an increasing or decreasing rate. For the first three trials the frequency of flicker increased until imperceptible, at which point the participant had to press either button on the response box when they could no longer see the light flickering. Another three trials were administered where the frequency of flicker decreased until perceptible. A further 20 trials were then administered where only one of the lights flickered and the participant was to press

either the left or right button which corresponded to the flickering light. The outcome measure used was the threshold level of flicker frequency (Hz) that the volunteer was able to perceive.

5.2.3.7 Mood Measure

Visual Analogue Mood Scale (VAMS): This was a traditional paper and pencil task which measured participants subjective mood, and has traditionally been used as an indicator of perceived drug effects (Bond and Lader 1974). The VAMS consists of 16 scales (100mm horizontal lines) with polar adjectives at either end (eg. alert – drowsy). Participants were instructed to mark a vertical line on the continuum which best represented how they felt at that moment in time. The scores for the 16 scales were collated into three factors (alertness, contentedness and calmness) according to Bond and Lader (1974), which were used as the mood measures in the current study.

5.2.4 Statistical Analysis

Data was analyzed using SPSS v15 (SPSS Inc. Chicago, IL). To determine whether there were any significant differences between the two treatment groups at baseline, t-tests and Pearson's Chi-Square tests were performed on demographic variables. The analysis and results of cognitive data are divided into two parts based on the study's objectives which; (1) explored the effects of E_2 on cognition after 28 days of treatment, and (2) investigated the interaction between E_2 treatment and the cholinergic system following a scopolamine challenge.

Part One – Effects of E_2 on Cognition: Separate mixed ANOVAs (with Huynh-Feldt adjustments where appropriate) were conducted on each of the cognitive outcome measures belonging to the 6 cognitive domains; declarative verbal memory and learning, verbal fluency, working memory, cognitive flexibility, attention, and psychomotor function and information processing. In the interest of minimizing the number of analyses conducted, the cognitive domains of working memory and information processing/psychomotor function which, within each respective domain, involved the same type of task but with different levels of difficulty (ie. 1- and 2-back tasks, SRT and CRT), were analyzed using 'Task Level' as a factor.

Furthermore, although participants were required to start the trial in the follicular phase of the menstrual cycle it was not possible to control the menstrual cycle phase at the day 28 assessment, thus 'Menstrual Cycle Phase' (based on self-report from the Menstrual Cycle Questionnaire) was included as a factor for all mixed ANOVAs. Follicular phase was classified as days between onset of menses and day 14, while the luteal phase was classified as day 15 up to the day prior to menstruation. Scores on cognitive measures, as well as the mood measure (VAMS), were the Dependent Variables, and the between-groups factors 'Treatment Group' (E_2/placebo) and 'Menstrual Cycle Phase' (follicular/luteal), as well as the within-groups factors 'Time' (baseline/post-treatment) and 'Task Level' (where relevant) were the Independent Variables. Within each cognitive domain, follow-up analyses were employed for significant main effects or interactions, with Bonferroni correction keeping Type I error within each domain at 0.05 (corrected p-values reported).

Part Two – Interaction between E_2 and the Cholinergic System following the Scopolamine Challenge: Prior to analysis of this data, cognitive baseline scores obtained on day 28 and day 31 of treatment were subtracted from each individual's post-injection day 28 and post-injection day 31 scores respectively, in order to obtain "change" scores. This method was chosen to minimize the number of factors within each analysis and to aid in the interpretation of results. The "change" scores reflected the change in performance from pre- to post-scopolamine and pre- to post-placebo (saline) challenge. Thus ordinarily, we would expect scopolamine change-scores to be negative (given that scopolamine is expected to impair cognition), and placebo difference-scores to be approximately zero. Separate mixed ANOVAs (with Huynh-Feldt adjustments where appropriate) were conducted for the same cognitive and mood outcome measures as in Part One (excluding verbal fluency and with the addition of the CFF task). Change scores on the cognitive measures, as well as those of the VAMS subscales, were the dependent variables, and the between-groups factors 'Treatment Group' (E_2/placebo) and 'Menstrual Cycle Phase' (follicular/luteal), as well as the within-group factors 'Drug Challenge' (scopolamine/placebo) and 'Task Level' (where relevant), were the Independent Variables. As in Part One, within each cognitive domain, follow-up analyses were employed for significant interactions (involving Treatment Group), with Bonferroni

correction keeping Type I error within each domain at 0.05 (corrected p-values reported).

Note that for both parts One and Two, in order to reduce skewness and meet the normality assumptions, outliers (identified using the boxplot inter-quartile range function in SPSS) that were deemed actual measures of performance and therefore part of the target population were transformed in order to reduce the skewness of the group's mean value. This was achieved by assigning a value of one unit more extreme than the next most extreme value in that group's population, as suggested by Tabachnick and Fidell (2001). Overall, 2.5% of the total values included in Part One and 3.3% of those included in Part Two were altered using this method. Analyses involving amended values were re-run using original data, to ensure that transformation did not substantially impact on the results.

Subject 15 was excluded from all working memory analyses and both subjects 15 and 28 were excluded from all attention analyses, due to a failure to demonstrate adequate understanding of one or more of the tasks in these cognitive domains (i.e. accuracy score of <50% under baseline testing conditions). Similarly, subjects 1, 10, 14 and 17 were excluded from the CFF analysis (part 2) due to an inability to adequately perform the task.

5.3 Results

5.3.1 Participants

Of the 34 participants who enrolled in the study a total of 30 completed both parts One and Two (3 people withdrew due to time constraints and 1 withdrew due to personal reasons) (see Figure 8). Estradiol treatment was well tolerated and no participant reported significant alterations to their normal menstrual cycle (it is unexpected that participant's cycle phase would change with such a short duration of treatment). The sample comprised women of various cultural backgrounds, although the majority were Australian-born, and all spoke fluent English. There were no significant differences in mean age, weight, predicted WAIS-R IQ (as determined by

the NART) or nationality found between the two groups (see Table 5). Independent samples *t*-tests conducted on each cognitive measure at baseline showed there were no significant differences in cognitive performance between the two groups. Furthermore, Pearson's correlations showed no significant relation between age and performance on any of the cognitive measures (data not shown). Seven participants from each of the two groups had previously been on the contraceptive pill, however it had been on average 1 year for the E_2 group and approximately 2.7 years for the placebo group since they stopped taking the pill. This difference was not statistically significant, nor was the average duration of taking the pill (see Table 5).

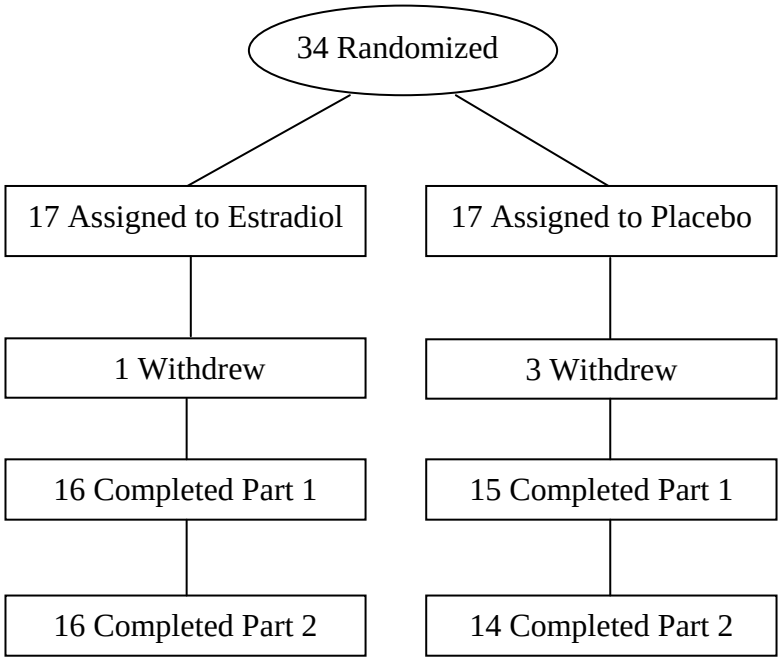

Figure 8. Flow Diagram of Study Population

Table 5. Demographic Characteristics

	Estrogen Group (N = 17)	Placebo Group (N = 17)	P value of t-test or χ^2
Mean Age (SD)	22.53 (3.69)	22.65 (5.22)	.940
Mean Weight (SD)	57.44 (11.44)	58.33 (14.06)	.848
Mean Predicted WAIS-R IQ (SD)	108.41 (7.19)	104.94 (6.36)	.146
Number Born in:			.467
Australia	7	10	
Fiji	0	1	
Asia	5	4	
Canada	2	0	
America	0	1	
France	1	0	
United Kingdom	1	0	
India	1	1	
Number with History taking the pill	7	7	1.00
Mean months duration on the pill (SD)	9.57 (10.16)	19.67 (26.94)	.418
Mean months since stopped the pill (SD)	12.36 (17.09)	32.07 (41.07)	.275
Menstrual cycle phase at day 28:			.886
Number in Follicular	10	9	
Number in Luteal	6	6	

Note: SD – standard deviation, WAIS-R IQ, Wechsler Adult Intelligent Scale Revised Intelligence Quotient.

5.3.2 Part 1: Effects of E_2 on Cognition

Analysis of the declarative verbal memory and learning domain revealed a significant interaction between Time and Treatment Group for the long-delay free recall measure ($F(1,27) = 4.79$, $p<.05$, partial $\eta^2 = 0.15$) (see Figure 9). Post hoc ANOVAs for the E_2 and placebo groups separately, showed a trend towards an improvement in performance for the E_2 group ($F(1,15) = 5.77$, $p=.06$, partial $\eta^2 = 0.28$), while there was no significant change in the placebo group (p= NS). There was a significant main effect of Menstrual Cycle Phase for List A total words ($F(1,27) = 7.83$,. $p<.01$, partial $\eta^2 = 0.23$), where participants performed better when in the follicular phase of the menstrual cycle. There were no other significant effects or interactions for the verbal learning and memory domain (see Table 6 for means and standard deviations of cognitive measures).

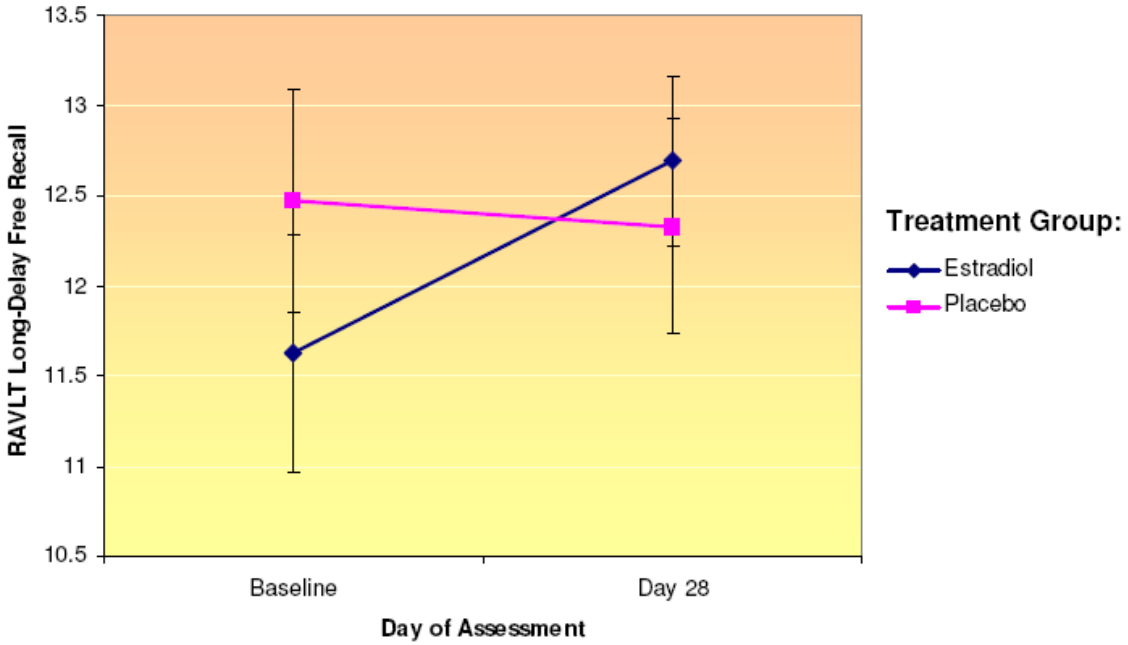

Figure 9. Effects of one month E_2 or placebo treatment on declarative verbal memory and learning in healthy young women (Part 1). Means ± SEM displayed for the RAVLT long-delay measure. A significant interaction between the E_2 and placebo group was found ($p<0.05$).

The working memory domain was analysed using 2 separate mixed ANOVAs with 'Task Level' (1-back/2-back) as a factor, for accuracy and RT. The ANOVAs revealed a significant interaction between Time and Treatment Group for accuracy of the n-back task ($F(1,26) = 6.02$, $p<.05$, partial $\eta^2 = .19$; see Figure 10) but not RT (p = NS). Post hoc analyses for accuracy data showed significant Time by Task Level interactions for both the E_2 group ($F(1,15) = 7.30$, $p<.05$, partial $\eta^2 = .33$) and the placebo group ($F(1,14) = 7.28$, $p<.05$, partial $\eta^2 = .34$). Further post hoc analyses for the 1-back and 2-back separately, showed that the estrogen group improved significantly on the 1-back (accuracy) measure of the n-back task ($F(1,15) = 6.41$, $p<.05$, partial $\eta^2 = .30$), while the placebo group displayed a significant drop in performance on the 2-back (accuracy) measure ($F(1,14) = 13.51$, $p<.01$, partial $\eta^2 = .49$). There were no other Time effects or interactions for the Working Memory domain (see Table 6).

Table 6. Means & Standard Deviations of Cognitive Scores at Baseline and Post-Treatment (Part 1)

Cognitive Measure	Estradiol Group (*N* = 16)		Placebo Group (*N* = 15)		P value:
	Baseline	Post-treatment	Baseline	Post-Treatment	Time x Group[1]
	M (SD)	M (SD)	M (SD)	M (SD)	
Verbal Memory & Learning					
RAVLT:					
List A total	57.19 (9.31)	57.00 (6.96)	56.73 (6.88)	58.53 (5.24)	.404
Long-delay free recall	11.63 (2.63)	12.69 (1.89)	12.47 (2.39)	12.33 (2.32)	.038*
Verbal Fluency					
COWAT:					
Total words	42.44 (8.91)	44.75 (8.58)	40.60 (11.48)	46.20 (10.41)	.118
Working Memory					
Visuospatial N-back:					
Correct: 1-back (%)	91.99 (5.37)	95.50 (2.81)	92.35 (6.13)	92.28 (6.10)	.021*
2-back (%)	88.93 (6.72)	87.99 (5.53)	89.24 (6.62)	83.96 (9.67)	
RT: 1-back (msec)	557.14 (177.80)	586.62 (184.94)	590.07 (185.64)	599.63 (175.20)	.095
2-back (msec)	604.14 (184.52)	658.70 (163.36)	673.39 (245.15)	643.69 (185.63)	
Cognitive Flexibility					
STROOP:					
Interference score	5.60 (7.75)	8.58 (7.25)	3.85 (7.05)	5.67 (7.59)	.522
Attention					
Digit Vigilance:					
Correct detection (%)	97.35 (2.65)	96.23 (4.10)	98.34 (2.69)	97.41 (3.20)	.615
RT (msec)	406.29 (44.85)	409.65 (46.73)	403.59 (50.83)	417.27 (33.50)	.454
Psychomotor Function & Information Processing					
RT: SRT (msec)	257.70 (35.22)	269.78 (36.84)	267.37 (44.82)	290.26 (60.39)	.983
CRT (msec)	395.90 (53.34)	405.25 (59.37)	426.52 (58.48)	429.51 (58.13)	

Note: [1]p value for Time by Treatment Group interactions only. RAVLT – Rey Auditory Verbal Learning Test, COWAT – Controlled Oral Word Association Test, SRT – Simple Reaction Time, CRT- Choice Reaction Time. *significance at the .05 level.

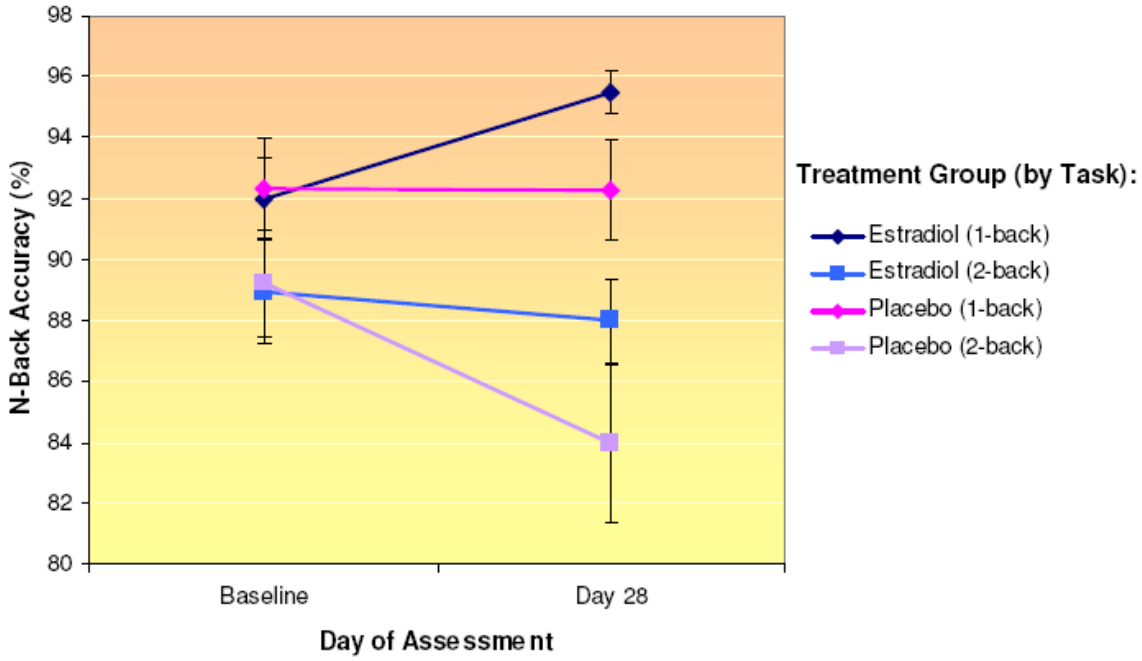

Figure 10. Effects of one month E_2 or placebo treatment on accuracy of the 1 and 2-back working memory measures in healthy young women (Part 1). Means ± SEM displayed for n-back percentage correct scores. A significant interaction between Time and Treatment Group was found ($p<0.05$). The E_2 group improved significantly on the 1-back ($p<.05$) while the placebo group showed a significant drop in performance on the 2-back task ($p<.05$).

A significant main effect of Time was found for the verbal fluency measure (COWAT total words; $F(1,27) = 17.12$, $p<.001$, partial $\eta^2 = .39$), where participants' performance improved over the 28 day treatment period. There was also a significant main effect of menstrual cycle phase for this measure ($F(1,27) = 8.15$, $p<.01$, partial $\eta^2 = .23$), where participants obtained higher scores when in the follicular phase of the menstrual cycle. There was no Time by Treatment Group interaction for the COWAT.

No significant main effects or interactions were observed for the domains of cognitive flexibility, attention or information processing/psychomotor function.

5.3.3 Part 2: Interaction between E_2 and the cholinergic system following the scopolamine challenge: Effects on Cognition

The analyses showed main effects of 'Drug Challenge' for a number of cognitive domains, where scopolamine compared to placebo (saline) was found to significantly impair performance within the domains of: declarative verbal memory and learning (Total words List A; $F(1,26) = 14.07$, $p \leq .001$, partial $\eta^2 = .35$, and long-delay free recall; $F(1,26) = 24.79$, $p < .001$, partial $\eta^2 = .49$), working memory (n-back accuracy; $F(1,25) = 59.45$, $p < 0.001$, partial $\eta^2 = .70$, and n-back RT; $F(1,25) = 30.91$, $p < .001$, partial $\eta^2 = .55$), attention (digit vigilance accuracy; $F(1,24) = 58.72$, $p < 0.001$, partial $\eta^2 = 0.71$, and digit vigilance RT; $F(1,24) = 19.11$, $p < 0.001$, partial $\eta^2 = 0.44$) and psychomotor function and information processing (RT: $F(1,26) = 74.96$, $p < 0.001$, partial $\eta^2 = 0.74$), but not cognitive flexibility or the CFF task of the psychomotor function and information processing domain (see Table 7 for means and standard deviations of cognitive change scores, see Appendix J for raw means and standard deviations of pre and post-challenge cognitive scores for an overall indication of actual performance).

A significant interaction between Drug Challenge, Treatment Group and Menstrual Cycle Phase was observed for the long-delay free recall measure of the RAVLT ($F(1,26) = 8.35$, $p < .01$, partial $\eta^2 = .24$, see Figure 11). Post hoc mixed ANOVAs for the two menstrual cycle phases separately revealed a trend towards an interaction between Drug Challenge and Treatment Group for participants who were in the follicular phase at post-treatment ($F(1,17) = 5.57$, $p = .06$, partial $\eta^2 = .25$), but not those in the luteal phase (p= NS). Further post hoc analyses of the placebo and E_2 groups separately (for participants in the follicular phase only) produced a significant main effect of the Drug Challenge for the placebo group ($F(1,8) = 16.31$, $p < .01$, partial $\eta^2 = .67$) but not the E_2 group (p= NS), suggesting that participants of the E_2 group who were in the follicular phase of the cycle performed similarly on the scopolamine challenge day as on the saline (placebo) challenge day.

Table 7. Means and Standard Deviations of Change Scores (Post-Challenge minus Baseline) for Cognitive Measures (Part 2)

Cognitive Measure	Estrogen group (n = 16) Scopolamine	Saline	Placebo group (n =14) Scopolamine	Saline	P value: Challenge	P value: Group x Challenge[1]
Verbal Memory & Learning						
RAVLT:						
List A total (trials 1-5)	-9.81 (5.95)	-2.00 (7.98)	-11.07 (11.54)	-2.29 (5.03)	.001*	.863
Long-delay free recall	-4.88 (2.73)	-1.31 (2.80)	-4.50 (3.74)	-.43 (2.03)	.000*	.664
Working Memory						
Visuospatial N-back:						
Correct: 1-back (%)	-8.93 (8.83)	-3.15 (3.93)	-10.25 (7.77)	-1.78 (5.62)	.000*	.321
2-back (%)	-12.41 (9.06)	2.08 (4.23)	-10.36 (6.03)	-3.00 (6.24)		
RT: 1-back (msec)	79.79 (72.62)	-10.17 (79.98)	109.99 (73.30)	26.83 (91.36)	.000*	.398
2-back (msec)	93.06 (129.64)	-25.09 (112.14)	100.94 (86.85)	15.92 (83.87)		
Attention						
Digit Vigilance[1]:						
Correct detection (%)	-7.31 (5.68)	.89 (3.44)	-14.02 (9.22)	-.78 (2.45)	.000*	.122
RT (msec)	41.34 (36.74)	-.79 (39.34)	50.35 (51.90)	15.28 (32.45)	.000*	.901
Cognitive Flexibility						
STROOP:						
Interference score	-1.80 (5.25)	-2.77 (6.46)	-1.23 (8.15)	-.44 (4.51)	.916	.658
Information Processing/ Psychomotor Speed						
RT: Simple (msec)	95.90 (69.07)	13.79 (25.69)	97.69 (74.02)	8.62 (24.14)	.000*	.314
Choice (msec)	58.44 (45.61)	6.37 (36.65)	82.36 (58.77)	-2.71 (37.79)		
CFF (Hz)	-4.07 (6.08)	.57 (4.13)	-.25 (8.04)	-1.67 (4.25)	.326	.151

Note: [1]p value displayed for Drug Challenge by Treatment Group only. CFF was analyzed separately to SRT & CRT. SRT- simple reaction time, CRT – choice reaction time, CFF – critical flicker fusion. * significant at the .001 level (see Appendix J for raw means and standard deviations of pre and post drug challenge cognitive scores).

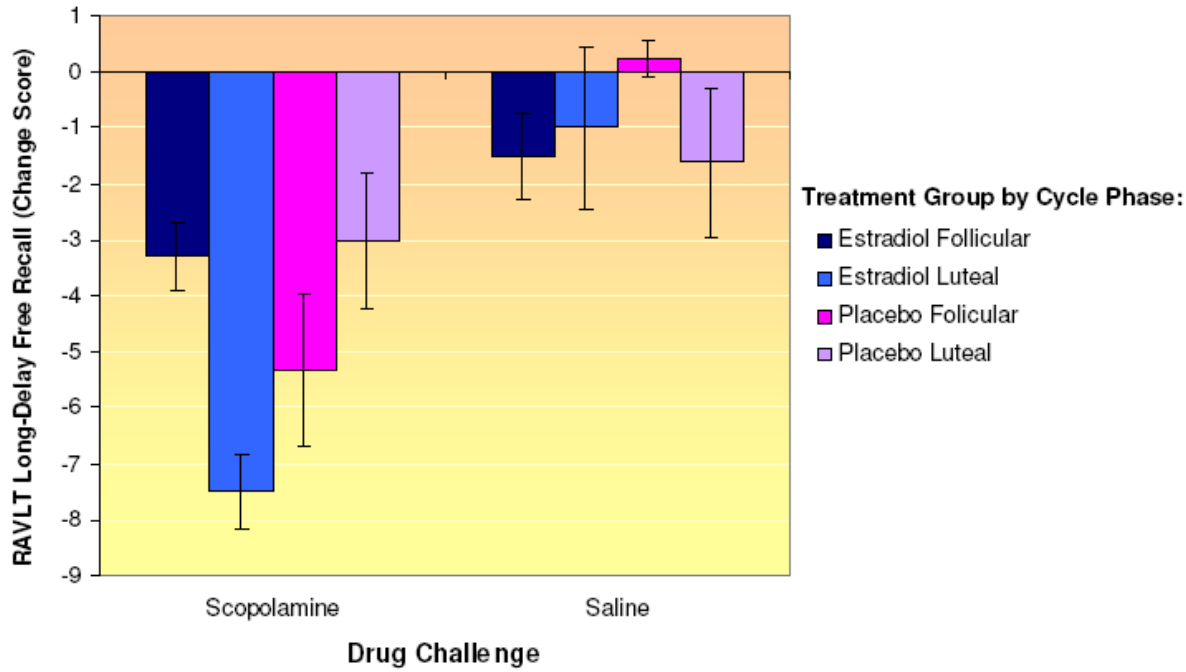

Figure 11. Effects of E_2 on declarative verbal memory and learning after a cholinergic challenge (Part 2). Means ± SEM of RAVLT long-delay free recall 'change' scores. A significant interaction was found between Drug Challenge, Treatment Group and Menstrual Cycle Phase ($p<.01$).

A significant main effect of Treatment Group was found for the digit vigilance accuracy measure of the attention domain ($F(1,24) = 6.15$, $p<.05$, partial $\eta 2 = .20$), where the E_2 group performed significantly better overall when compared to the placebo group. There were no Treatment Group by Drug Challenge interactions or Treatment Group by Drug Challenge by Menstrual Cycle Phase interactions for the attention domain.

Significant Drug Challenge by Menstrual Cycle Phase interactions were observed for the RT measure of the working memory domain ($F(1,25) = 4.69$, $p<.05$, partial $\eta^2 = .16$) and for information processing/psychomotor function ($F(1,26) = 10.21$, $p<.01$, partial $\eta^2 = .28$). There were no interactions with Treatment Group for these measures, thus no post hoc analyses were conducted.

There were no Drug Challenge by Treatment Group interactions for the remaining measures corresponding to the domains of: cognitive flexibility and psychomotor

function and information processing, suggesting that task performance of these domains were similar for both groups under the drug-challenge conditions. Furthermore there were no main effects of or interactions with Menstrual Cycle Phase for the domains of: verbal fluency, attention and cognitive flexibility, in Part 2 of the analyses.

5.3.4 Mood Measure

Part 1: Effects of Short-term E_2 Treatment on Mood: There were no Time by Treatment Group interactions for any of the 3 subscales of the VAMS (see Table 8 for means and standard deviations of baseline and post-treatment scores).

Table 8. Means and Standard Deviations for Mood Factors (Part 1)

	Estrogen Group (*n*=16)		Placebo Group (*n*=14)		P
Measure	Baseline	Post-treatment	Baseline	Post-treatment	value[1]
VAMS					
Alertness	377.37 (171.84)	376.81 (176.09)	345.20 (164.07)	392.20 (152.94)	.383
Contentedness	140.69 (67.31)	174.87 (83.67)	153.53 (86.87)	169.40 (91.33)	.787
Calmness	65.00 (32.62)	76.31 (34.05)	63.87 (39.88)	63.73 (27.46)	.576

Note: [1]p value for Time by Treatment Group interaction, VAMS- Visual Analogue Mood Scale.

Part 2: Interaction Between E_2 and the Cholinergic System Following the Scopolamine Challenge: Effects on Mood: There were no Drug Challenge by Treatment Group interactions, nor were there any main effects of Treatment Group for the VAMS measures in the Part Two analysis. There was however a significant main effect of Drug Challenge for alertness ($F(1,26) = 53.97$, $p<.001$, partial $\eta^2 = .68$) and contentedness ($F(1,26) = 20.18$, $p<.001$, partial $\eta^2 = .44$) but not calmness (p = NS), where overall alertness and contentedness for both groups was worse after scopolamine compared to saline (see Table 9 for means and standard deviation of change scores for the mood measures, see Appendix K for raw means and standard deviations of pre and post-drug challenge mood scores for an overall indication of effects on mood).

Table 9. Means and Standard Deviation of Change Scores for Mood Factors (Part 2)

Measure	Estrogen Group (*n*=16) Scopolamine	Saline	Placebo Group (*n*=14) Scopolamine	Saline	P value[1]
VAMS					
Alertness	267.94 (156.44)	12.75 (113.70)	240.00 (135.81)	-5.64 (92.11)	.852
Contentedness	54.25 (43.06)	-21.06 (57.91)	51.36 (84.25)	-20.07 (27.05)	.756
Calmness	-13.31 (29.36)	-9.88 (19.30)	-6.14 (24.68)	-4.86 (11.14)	.944

Note: [1]p value for Drug Challenge by Treatment Group interaction, VAMS- Visual Analogue Mood Scale. Lower scores represent greater alertness, contentedness and calmness.

5.3.5 Subjective Ratings of Peripheral Effects Post Drug Challenge

Analysis of subjective ratings of adverse effects post- drug challenge was accomplished using 2 (Drug Challenge) by 2 (Time; pre and post drug administration) mixed ANOVAs for each of the ten items of the Adverse Symptoms Checklist. 'Time' was included as a factor because difference scores were not able to be calculated given that the level of measurement was based on a 4-point scale as described earlier (responses of 'unsure' were excluded from the analysis). Given the large number of variables within this measure, α was set at .01 level. There were no significant differences found in the rating of adverse reactions between the two treatment groups. The results showed significant Drug Challenge by Time interactions for the items: dizzy ($F(1,24) = 52.92$, $p<.001$, partial $\eta^2 = .69$), blurred vision ($F(1,26) = 38.24$, $p<.001$, partial $\eta^2 = .60$), nausea ($F(1,21) = 8.64$, $p<.01$, partial $\eta^2 = .29$) and dry mouth ($F(1,24) = 59.08$, $p<.001$, partial $\eta^2 = .71$). On average participants reported not experiencing these symptoms at all during the placebo challenge while after scopolamine administration participants, on average, reported feeling moderately dizzy, had somewhat blurred vision and had a moderately dry mouth (see Appendix L for means and standard deviations of adverse symptoms ratings).

5.4 Discussion

The current study examined the cognitive effects of one month E_2 treatment and its interaction with the cholinergic system in healthy young women. Our findings showed that E_2 treatment: (1) enhanced accuracy on a measure of working memory,

had a tendency to improve long-term verbal memory, but had no effect on verbal fluency, cognitive flexibility, attention or information processing and psychomotor functioning, and (2) generally did not protect against the scopolamine-induced impairments in cognitive function. These effects were observed independent of mood.

To our knowledge this is the first study to have investigated the neurocognitive effects of short-term E_2 treatment in healthy young (age 18-38) pre-menopausal women. The majority of past research that examined the link between estrogen and cognition in this age cohort used the menstrual cycle phase as a methodological construct. Some researchers previously found verbal fluency, articulation, verbal working memory, visual memory, speed of information processing and psychomotor functioning to be better during the high estrogen (either pre-ovulation or mid-luteal) phases of the menstrual cycle (Hampson 1990a; Hampson 1990b; Hampson and Kimura 1988; Keenan et al 1992; Phillips and Sherwin 1992b; Rosenberg and Park 2002). However, findings are mixed as research has also failed to find significant effects of menstrual cycle phase on various cognitive processes, including verbal fluency, learning, spatial skills, information processing, attention and working memory (Epting and Overman 1998; Gordon and Lee 1993; Hampson 1990b; Kasamatsu et al 2002; Phillips and Sherwin 1992b; Rosenberg and Park 2002; Ussher and Wilding 1991), which is partly in line with our findings of no effects of E_2 treatment or menstrual cycle phase on the majority of cognitive domains assessed.

The finding of improved working memory with E_2 treatment in Part 1 is difficult to interpret, given that the limited previous research which specifically examined the effects of estrogen on this cognitive domain has been inconsistent. For example, one study found verbal working memory to be better during the high estrogen (pre-ovulation) phase of the menstrual cycle (Rosenberg and Park, 2002), while another study reported no difference in working memory across menstrual cycle phases (Phillips and Sherwin, 1992). Worth noting, it is difficult to compare the current findings to that of menstrual cycle research given that the E_2 administered in the current study may have altered participants' menstrual cycles, based on research that

has investigated contraceptive effects where breakthrough bleeding and spotting can occur (Andolsek 1992; Reisman et al 1999).

The improvement in spatial working memory in the current study is also generally inconsistent with the postmenopausal HT literature. Surprisingly, few studies have specifically examined "visuospatial" working memory (Duff and Hampson 2000; Joffe et al 2006; Smith et al 2006), with only one study finding significant improvements following ET (Duff and Hampson 2000). The majority of previous research in this area has failed to find an effect of HT on working memory of any modality (Alhola et al 2006; Galen Buckwalter et al 2004; Janowsky et al 2000; Joffe et al 2006; Low et al 2006; Maki et al 2001) or tasks requiring visuospatial skills (Alhola et al 2006; Almeida et al 2006; Galen Buckwalter et al 2004; Joffe et al 2006; Kampen and Sherwin 1994; Maki and Resnick 2000; Maki et al 2001; Phillips and Sherwin 1992a; Resnick et al 1998; Shaywitz et al 2003; Yaffe et al 2006). Despite this, a few researchers have found significant positive effects of either ET or HT on verbal working memory (Duff and Hampson 2000; Keenan et al 2001; Miller et al 2002) and general spatial tasks (Duka et al 2000; Verghese et al 2000), further demonstrating inconsistency within the literature. It should be noted that the significant effect on working memory in the current study is not likely to be due to an accuracy-reaction time trade-off, given that the reaction time component was not significantly different between the two groups for this task. In addition it may be that E_2's effects are specific to a low working memory load, given that the 2-back was not significantly affected. Readers should also be mindful that the improvement in this task was quite small (effect size = .30), where mean performance went from 92% to 95%, thus it is unlikely that a difference would be observed in real-world functioning for healthy young women. Nevertheless, this small effect may be due to the already high level of functioning (near ceiling levels), thus finding has implications for the treatment of cognitive deficits in schizophrenia given that spatial working memory is consistently found to be markedly impaired in individuals with this illness (for review see Piskulic et al 2007).

The large body of literature addressing the cognitive effects of ET/HT in postmenopausal women, while grossly inconsistent, shows verbal memory to be the

only domain with some reliable tendency towards better performance with estrogen (Maki et al 2001; Phillips and Sherwin 1992a; Resnick et al 1998; Sherwin 1988; Sherwin 1998; Sherwin 1999; Sherwin 2003; Sherwin 2006). Therefore, the finding that the E_2 group tended to remembered more words on the RAVLT long-delay verbal recall measure after 28 days treatment, is in line with previous research. However, this finding was only at the trend level after controlling for multiple comparisons, and no treatment effects were found for the other measure of the declarative verbal memory and learning domain (RAVLT total words list A trials 1-5).

One possible explanation for the current finding of no significant effects on most of the cognitive tasks may relate to the fact that participants were young pre-menopausal women. A significant improvement in cognitive function was not anticipated or hypothesized given that participants were healthy and already functioning at a high level, with no cognitive impairments that required the need for pharmacological treatment. It may be that E_2 treatment is only effective when there is a deficit in cognitive functioning, such as that seen in schizophrenia or the cognitive deterioration associated with normal aging. However, this theory is challenged by the current study's finding of enhanced working memory, suggesting that some cognitive domains are capable of being enhanced with E_2 even in young women. Differences in circulating endogenous E_2 levels between young and aged women may also account for discrepancies between previous research and the current study. The majority of previous research investigating the effects of ET/HT used postmenopausal women, where the addition of 100µg transdermal E_2 for example, would bring circulating E_2 levels up to that seen during the early-mid follicular phase in healthy cycling women (approx. 257-694 pmol/l) (Dören 2001; Leonard 2004). However, in the current study, because the sample comprised healthy young women, the addition of 100µg transdermal E_2 treatment is thought to raise plasma E_2 to above physiological levels (minimum approx. 400 pmol/l during the early follicular phase, maximum approx. 2121 pmol/l during ovulation) (Dören 2001; Leonard 2004). Therefore it is possible that in the current study E_2 exceeded an 'optimal level' where an intermediate level of E_2 may produce the best cognitive result.

The duration of E_2 treatment may also in-part explain the differences between our findings and past research. Verbal memory, as well as speed of information processing and vigilance, have previously been reported to improve in experimental studies of postmenopausal women receiving ET/HT for at least two months duration (Dumas et al 2008; Hogervorst et al 1999; Joffe et al 2006; Linzmayer et al 2001; Phillips and Sherwin 1992a; Saletu 2003). Similarly, improvements in verbal memory, working memory and global cognitive functioning have also been reported in prospective studies where post-menopausal women were re-tested after receiving ET/HT for a number of years (Jacobs et al 1998; Rice et al 2000; Stephens et al 2006).

In the second part of the study we examined the interaction between E_2 and the cholinergic system, and found no protective or attenuating effect of E_2 treatment on cognitive function following the scopolamine challenge in our sample of healthy young women. Although the results suggested E_2 may have partially attenuated the effects of scopolamine on the long-delay free recall measure of the declarative verbal memory and learning domain, one should afforded little weight to this result given the initial interaction was of borderline significance, and specific to the menstrual cycle phase at the time of assessment (ie. the follicular phase only). One should also be mindful that the number of participants in the post hoc analyses were quite small (E_2 N = 10, placebo N = 9), thus a significant impairment in performance after the scopolamine as compared to the saline challenge may have been observed for the E_2 group had the sample been larger. Having said this however, the effect of scopolamine on the placebo group's performance was large enough to produce a significant result.

The current findings differ to those of two recent studies that have similarly examined the effects of estrogen on scopolamine-induced cognitive deficits, although in a different population, postmenopausal women (Dumas et al 2006; Dumas et al 2008). Dumas and colleagues (2006) first reported ET to significantly attenuate the cognitive-impairing-effects of scopolamine on a measure of attention and psychomotor function but not other cognitive measures. More recently they reported

attenuation of episodic memory in younger postmenopausal women but impairments in older postmenopausal women (Dumas et al 2008). These incongruent findings may be related to differences in study design, sample of women tested, methodology and analysis. Most notably, the latter studies by Dumas and colleagues (2006; 2008) examined the effects of E_2 in postmenopausal women while the current study involved women of child bearing age with normal menstrual cycles. As mentioned earlier it is expected that the addition of 100µg transdermal E_2 treatment in this group of women would raise plasma estradiol to above physiological levels (minimum approx. 109 pg/ml during the early follicular phase, maximum approx. 578 pg/ml during ovulation) (Leonard 2004), again suggesting that an optimum level of estrogen for behavioral effects may have been exceeded and therefore did not protect overall cognitive functioning in the current study. Secondly Dumas and colleagues (2006; 2008) examined the effects of three months E_2 treatment compared to one month as in the current study, suggesting a longer duration of treatment may be needed to build a defense against the cognitive-impairing effects of scopolamine. Worth noting is that Dumas and colleagues (2006) initially found significant effects on attention measures, but were unable to replicate these findings in their later study (Dumas et al 2008), further testifying to the inconsistency in this area of research.

The use of scopolamine in research on healthy subjects is a well established pharmacological tool for disrupting cholinergic functioning and inducing cognitive deficits similar to that seen in dementia and AD (Ebert and Kirch 1998). Few experimental studies of short-term ET in AD (a neurodegenerative disorder characterized by cholinergic dysfunction) have revealed significant positive effects (Asthana et al 2001; Asthana et al 1999), with the growing consensus being that ET initiated after onset does not alleviate cognitive deficits, nor does it slow the rate of deterioration (Mulnard et al 2000; Pinkerton and Henderson 2005; Shaywitz and Shaywitz 2000; Shumaker et al 2004; Thal et al 2003). However, a large body of epidemiological research does support a possible protective effect of long-term ET or HT use (ranging from 1 to >10 years), in regards to reducing the risk of developing AD in later life (Costa et al 1999; Paganini-Hill and Henderson 1996; Tang et al 1996; Waring et al 1999; Zandi et al 2002). Therefore, although one month of E_2 did not protect against scopolamine in this study one cannot rule out a possible protective

effect with longer duration ET, which is supported by the findings of Dumas and colleagues (2006; 2008).

One limitation of the current study was that plasma estrogen levels were not measured. Despite this all participants were deemed physically healthy by a physician and participants with a history of uterine or endocrine abnormalities were excluded, thus E_2 levels were expected to be within the normal range. However, plasma estrogen levels would have been useful to aid in evaluating menstrual cycle phase in addition to verbal reports, and to ensure estrogen levels increased following E_2 treatment. Although all women were required to start the study in the follicular phase (days 1-10 based on day 1 of menstruation), it was not possible to control the cycle phase at post-treatment. In order to investigate whether fluctuations in endogenous estrogen levels would interact with E_2 treatment effects, we included menstrual cycle phase as a factor in the cognitive analyses. The majority of cognitive domains were unaffected by menstrual cycle phase in Part 1 of the current study. However, the effects of E_2 treatment of performance of the RAVLT long-delay free recall measure after scopolamine in comparison the placebo (saline), did depend on the menstrual cycle phase participants were in. Specifically, E_2 treatment partially protected against the scopolamine-induced deficit for women who were in the follicular (low estrogen) phase only. This suggests that for women in the E_2 group who were in the luteal phase of the menstrual cycle, the level of E_2 had possibly exceeded the optimal threshold for a protective effect on long-term memory. However, as mentioned earlier this finding should be interpreted with caution.

Secondly, it should be noted that lack of significant protective effects of E_2 treatment on the cholinergic challenge may have also been related to the sample size, which was relatively small and only capable of detecting low (0.14) to moderate (0.40) effects with approximately 80% power (Cohen 1992). Thus, it is possible that smaller effects (where η^2 = 0.01 to 0.06) of estrogen treatment on the cholinergic system may have been detected with a larger sample (approximately 393 to 64 participants respectively), yet effects of this size are unlikely to be of any physiological importance or to impact on real-world cognitive functioning.

A significant treatment group effect on attentional processing was observed in Part Two of the analysis (irrespective of the challenge), which was surprising given no significant differences were found in this cognitive domain for the Part One analysis, highlighting the inconsistent effects of E_2 treatment even within the current study. Nevertheless, irrespective of the drug challenge, the estrogen group performed significantly better overall on the sustained attention task compared to the placebo group. While this is in line with some previous studies in postmenopausal women taking ET (Carlson and Sherwin 1998; Friebely et al 2001; Saletu 2003; Smith et al 2001a), it is inconsistent with the large number of other studies that have found no effects of estrogen on measures of attention (Alhola et al 2006; Barrett-Connor and Kritz-Silverstein 1993; Joffe et al 2006; Kampen and Sherwin 1994; Keenan et al 2001; Maki et al 2001; Morse and Rice 2005; Phillips and Sherwin 1992a; Polo-Kantola et al 1998; Resnick et al 1998; Schmidt et al 1996; Shaywitz et al 2003; Verghese et al 2000; Yaffe et al 2006). Worth noting is that in Part Two of the analysis, change in performance was relative to baseline pre-drug scores recorded earlier that same day, suggesting performance may have improved due to an effect of E_2 on practice, learning or a non-specific interaction with the drug challenges, thus this finding should be interpreted with caution.

In light of the implications of the current finding, that E_2 had minimal effects on cholinergic-modulated cognitive processes, it is possible that E_2 may modulate cognitive processes via other neurotransmitter systems, such as the dopaminergic system. Animal research has found ET to: (1) attenuate the gonadectomy-induced reduction in axonal density and tyrosine hydroxylase immunoreactivity of dopamine neurons in the prefrontal cortex of male rats (Kritzer 2000; Kritzer et al 2007), (2) be associated with a higher 3,4-dihydroxyphenylalanine (DOPAC)/dopamine ratio in the medial prefrontal cortex of rats following introduction to a novel environment (Handa et al 1997), and (3) increase striatal dopamine D_1 and D_2 receptor density in male and female animals (for reviews see Di Paolo 1994; Dluzen 2005). In addition, human studies have shown better dopaminergic responsivity and increased serum dopamine levels in post-menopausal women taking ET (Craig et al 2004; Zarate et al 2002). One study has investigated the relationship between menstrual cycle phase and D_2 receptor density in the putamen and cerebellum using single PET and

raclopride, but failed to find any significant results (Nordstrom et al 1998). Given that dysfunction of the mesoprefrontal cortical dopamine system has been specifically linked to cognitive impairment in schizophrenia, plus the prominent role of dopamine in the pathophysiology of the illness, further investigation into the modulatory effects of estrogen on this neurotransmitter system is needed.

Similarly, the serotonergic system has also been proposed as a possible modulatory mechanism for estrogen's actions on cogniton. Specifically, research has shown ET to; (1) increase the density of 5-HT_{2A} receptor binding in numerous brain regions, including the frontal cortex, of animals and humans (Cyr et al 1998; Fink et al 1996; Kugaya et al 2003; Moses et al 2000; Sumner and Fink 1995), (2) increase 5-HT_{2C} receptor mRNA levels in the hypothalamus and midbrain of OVX rats (Zhou et al 2002) (3) decrease density of serotonin reuptake transporter mRNA in the dorsal raphe nucleus of OVX macaques (Bethea et al 2002; Pecins-Thompson et al 1998), and (4) decrease 5-HT_{1A} receptor binding and mRNA levels in the hippocampus of OVX female rats (Birzniece et al 2001; Osterlund et al 2000). This latter finding is consistent with the reports of a negative relationship between hippocampal 5-HT_{1A} receptor binding/activation with memory and learning (Carli et al 1995; Yasuno et al 2003). In addition, young and post-menopausal women have been shown to demonstrate greater impairments in declarative memory than men when 'tryptophan', the central serotonin precursor, is depleted causing a reduction in 5-HT synthesis (Sambeth et al 2007). Similarly, central 5-HT tone has been found reduced in post-menopausal women who were ET naïve, whereas 5-HT tone was preserved in ET-users (van Amelsvoort et al 2001). A study in healthy women of child-bearing age has also shown platelet serotonin transporter binding and 5-HT_{2A} receptor binding to fluctuate with the menstrual cycle, where binding was lowest during the mid-luteal phase (Wihlback et al 2004). Overall, given that the exact role of the serotonergic system in learning and memory functions has yet to be fully characterized, plus taking into consideration the complex interactions serotonin has with the cholinergic, glutamatergic and GABAergic systems, interpretation of the modulatory effects of estrogen on the serotonergic system in relation to cognition is premature at this stage.

5.5 Conclusion

In conclusion, our findings suggest that short term (one month) E_2 treatment had a selective enhancing effect on working memory with a trend towards enhanced delayed verbal recall, but with no significant effects on the majority of cognitive domains assessed. Estradiol treatment appears to have attenuated the scopolamine-induced deficit in delayed verbal recall but not general declarative verbal learning and memory, or any of the other cognitive domains including: verbal fluency, working memory, attention, cognitive flexibility or psychomotor function/information processing. Thus in summary, short term E_2 treatment in this sample did not protect against (or attenuate) the cognitive deficits induced by scopolamine. These findings add to the already largely inconsistent literature and highlight the complex effects of ET on cognitive function, which may be further influenced by age, endogenous estrogen levels and duration of treatment. Insight into the cognitive effects of E_2 treatment in young healthy pre-menopausal women has implications for the treatment of cognitive dysfunction in women with schizophrenia, with the current results leaning towards the possibility that improvement in overall cognition is unlikely but that select domains of cognitive functioning may be significantly affected with adjunctive E_2 treatment. However, this is highly speculative given the basic differences in demographic variables and level of functioning between healthy women and women with schizophrenia, which highlights the need for investigation into the neurocognitive effects of E_2 treatment in this clinical population. In addition the findings suggest that E_2's short-term effects on cognitive functions in young women may not be primarily mediated via interaction with the cholinergic system. Further research into the diversity of estrogen's underlying cellular mechanisms and its interaction with other neurochemicals is needed to elucidate the role of estrogen in neuroprotection and cognitive enhancement in both young and postmenopausal women.

SECTION THREE:
COGNITIVE DYSFUNCTION IN SCHIZOPHRENIA:
IS ESTRADIOL A TREATMENT OPTION?

Chapter 6

Experiment Two: The Effects of Adjunctive Estradiol Treatment on Cognition in Women with Schizophrenia

"…….. there has been a true renaissance in the appreciation of the importance of cognition as the core of schizophrenia, from which most, if not all, aspects of the syndrome originate (Meltzer et al 1999)".

6.1 Introduction

6.1.1 Cognitive Impairment in Schizophrenia

Schizophrenia is a severe psychotic disorder that is prevalent in approximately 1% of the population world-wide, and is characterized by fundamental disturbances in thinking, perception, emotions and behavior (Rossler et al 2005). In 1896 Emil Kraeplin first characterized schizophrenia which he initially called "dementia praecox" (Kraepelin 1896, In Rossler et al 2005). While the symptoms of the disorder were noticeably diverse across the various sub-classifications (ranging from paranoia to catatonia), Kraeplin believed they all shared the common core feature of an early onset and progressive intellectual deterioration (Davison and Neale 2001). However from the early 50s to late 80s the disorder was increasingly characterized by the presence of hallucinations and delusions which became the focus of diagnosis and treatment, while recognition of the cognitive deficits fell by the wayside. Despite this, the 90s brought forth a newfound wealth of interest surrounding the impact of cognitive impairment associated with this disorder. It is now well-known that the characteristics of schizophrenia include pervasive impairments in cognitive functions, where as many as 98% of individuals with schizophrenia display a significant cognitive function decrement, based on predicted premorbid cognitive functioning (Keefe et al 2005). This impairment ranges between moderate to severe in magnitude and generally persists throughout the course of the illness, even if positive symptoms have been successfully treated (Heaton et al 2001). A review published a decade ago quantitatively examined 204 studies that had assessed performance on measures of verbal and non-verbal memory, motor performance, attention, general intelligence, spatial ability, language, executive functioning and tactile identification/discrimination in schizophrenia patients (Heinrichs and Zakzanis 1998). This study concluded that cognitive impairment in schizophrenia is wide-spread with varying degrees of impairment in all domains of cognition. More

recent reviews and a meta-analysis further confirmed the presence of significant impairments in all areas of cognitive functioning (Barch 2005; Bowie and Harvey 2005; Fioravanti et al 2005; Lewis 2004). In addition, a number of other large-scale reviews and meta-analyses have been conducted on independent cognitive domains including working memory (Lee and Park 2005; Piskulic et al 2007; Spindler et al 1997), recall and recognition (Aleman et al 1999), verbal (declarative) memory (Cirillo and Seidman 2003; Toulopoulouand and Murray 2004), selective attention (Henik and Salo 2004), executive function (Li 2004; O'Grada and Dinan 2007) and psychomotor processing speed (Morrens et al 2007), all of which consistently report serious impairments in performance of schizophrenia patients when compared to healthy controls. Working and verbal memory are probably the most frequently examined processes in schizophrenia, given that they tend to be more severely impaired than other cognitive domains (for reviews see Bowie and Harvey 2005; Cirillo and Seidman 2003; Lee and Park 2005; Piskulic et al 2007; Spindler et al 1997). Although there was initially some debate as to the nature of the working memory deficit (i.e. whether performance related to the central executive model of dysfunction), findings currently suggest that multiple systems of working memory are impaired, including spatial, object and verbal forms (Lee and Park 2005; Piskulic et al 2007; Spindler et al 1997). Between these sub-systems, visuospatial working memory appears to be more severely impaired than other working memory modalities (Lee and Park 2005).

Not surprisingly, a number of factors can influence the outcome of research examining cognitive deficits in schizophrenia, in particular gender, type of diagnosis, duration of illness, age of illness onset, level of education and socio-economic status, which should be taken into consideration when interpreting results. Given that impairments in cognitive processes are well-established, and that cognitive deficits are globally acknowledged as a significant symptom dimension inherent to schizophrenia, it will not be reviewed further (for reviews see Aleman et al 1999; Barch 2005; Bowie and Harvey 2005; Cirillo and Seidman 2003; Fioravanti et al 2005; Heinrichs and Zakzanis 1998; Henik and Salo 2004; Lee and Park 2005; Lewis 2004; Li 2004; Morrens et al 2007; O'Grada and Dinan 2007; Piskulic et al 2007; Spindler et al 1997; Toulopoulouand and Murray 2004).

Although positive symptoms have thus far been the primary target of current pharmacological treatments for schizophrenia, research suggests that cognitive deficits are the most 'functionally limiting' symptom dimension, and have the greatest impact on illness outcome in terms of employment status, social functioning and independent living (Breier et al 1991; Green 1996; Green et al 2000; Lysaker et al 1995; Velligan et al 1997). These indicies of functional outcome have been specifically linked to performance on measures of working memory, declarative memory, vigilance and executive functions (for review see Green 1996; Green et al 2000; Green et al 2004). Furthermore, research shows that cognitive deficits are commonly present from the first episode of psychosis (Addington and Addington 2002; Ayres et al 2007; Bilder et al 1992; Fitzgerald et al 2004), with some researchers suggesting cognitive deficits may even pre-date the appearance of psychotic phenomena (Brewer et al 2005; Erlenmeyer-Kimling et al 2000). More recent research shows that degree of cognitive impairment at the initial onset of first episode psychosis can be predictive of functional outcome at 1-year follow-up (Tabares-Seisdedos et al 2008). Since the re-emergence of cognitive impairment as an independent, unequivocal core feature of schizophrenia, the need for effective treatment in alleviating cognitive deficits has become much sought after.

6.1.2 Treatments Examined for Improving Cognitive Deficits in Schizophrenia

Numerous research groups, and pharmaceutical companies, have investigated the effects of atypical antipsychotics on cognitive impairment in schizophrenia. In particular clozapine, risperidone and olanzapine have been found to improve cognitive performance when compared to conventional antipsychotics and baseline cognitive functioning (Bilder et al 2002; Borkowska et al 2002; Cuesta et al 2001; Gur et al 2003; Harvey et al 2003; Keefe et al 2007a; Keefe et al 2007b; Kim and Kang 2004; Manschreck et al 1999; Potkin et al 2001; Riedel et al 2007; Wagner et al 2005; Zhong et al 2006). Keefe and colleagues (1999) performed a meta-analysis, and while acknowledging inconsistencies in the literature, concluded that atypical antipsychotics generally do improve cognitive functioning, particularly verbal fluency, executive function, fine motor skills and speed of information processing.

Less consistent improvements have also been observed in verbal learning and memory, visual memory, working memory and attention. However, in the instances where cognitive deficits improved with antipsychotic medication, cognitive performance remained well below normal levels of functioning. More recently other atypical antipsychotics that have also been found to improve at least some aspect of cognitive functioning include quetiapine (Fleming et al 2001; Good et al 2002; Keefe et al 2007a; Keefe et al 2007b; Kivircik Akdede et al 2005; Purdon et al 2001; Riedel et al 2007; Sax et al 1998; Velligan et al 2002; Velligan et al 2003; Voruganti et al 2007; Zhong et al 2006), ziprasidone (Harvey 2003; Harvey et al 2004; Keefe et al 2007a) aripiprazole (Cornblatt et al 2002; Gupta and Masand 2004; Kern et al 2006) and perphenazine (Keefe et al 2007a). However, one should be cautious when viewing this area of literature given methodological limitations that can impact on interpretation of results, such as pre-morbid cognitive functioning, concomitant medications, sample size and length of treatment. Furthermore, although review of the literature supports atypicals as having beneficial effects on cognitive impairment (Harvey and McClure 2006; Kasper and Resinger 2003; Meltzer and McGurk 1999), improvements tend to be modest and normal levels of cognitive functioning are rarely restored, thus it is imperative that the search for effective treatment for cognitive deficits in schizophrenia continues.

Given that many of the cognitive domains affected in schizophrenia are known to require cholinergic innervation, some researchers have suggested acetycholine esterase inhibitors (AChEI) and muscarinic and nicotinic receptor agonists as possible adjunctive treatment for cognitive dysfunction in schizophrenia (for review see Ferreri et al 2006; Friedman 2004). Open-label trials of the AChEI donepezil suggest possible beneficial effects on certain cognitive domains, including verbal recall, visual memory and processing speed, as well as a measure of global cognition (Buchanan et al 2003; Stryjer et al 2003). Methodologically controlled clinical trials of donepezil as an adjunct to antipsychotics however, have generally failed to find significant effects (Freudenreich et al 2005; Friedman et al 2002; Mazeh et al 2006), with only one study finding a significant improvement in declarative verbal learning and memory (Erickson et al 2005). In addition, an fMRI study found that 12 weeks of adjunctive donepezil treatment normalized frontal and cingulate cortical activation

of schizophrenia patients during a verbal fluency task in comparison to placebo treatment, although no change was observed on behavioural task performance (Nahas et al 2003). A number of trials have also been conducted on the AChEI rivastigmine. An open-label trial in chronic schizophrenia patients with co-morbid dementia resulted in improved MMSE scores after 12 weeks of treatment with 9mg/day oral rivastigmine (Mendelsohn et al 2004). A cross-over event-related potentials (ERP) trial, conducted in schizophrenia patients without co-morbid dementia, showed rivastigmine to increase the frontal P2a amplitude but decrease the posterior temporal N2b negativity during a recognition memory task (Guillem et al 2006). This frontal ERP activity has been suggested to correspond to processing the salience of the stimulus as well as inhibition of irrelevant stimuli (Guillem et al 2003). Conversely, the N2b activity is said to represent categorization and reactivity to the stimulus, suggesting adjunctive rivastigmine altered information processing cascades (Guillem et al 2003). However, no significant improvements were found in the performance of cognitive tasks (Guillem et al 2006). Similarly, a recent placebo-controlled double-blind study and another randomized cross-over trial have failed to find any effects of 3 or 6 months adjunctive rivastigmine treatment on various cognitive measures (Chouinard et al 2007; Sharma et al 2006). One possible cause for these mixed findings may be related to the type of antipsychotic administered in conjunction with the AChEI. Animal studies have shown that certain antipsychotic medications increase cortical and hippocampal ACh release (Chung et al 2004; Ichikawa et al 2002a; Ichikawa et al 2002b; Li et al 2005; Li et al 2003), mechanisms associated with cholinergic-mediated cognitive functioning (see Chapter 3 for review). Thus, it is possible that the doses of AChEI used in the above clinical trials may have resulted in too much ACh in the cortex to produce positive effects. This is in line with animal research that has shown modulation of cognitive performance with cholinomimetic drugs tends to be of an inverted U-shaped response (Santucci et al 1991). While higher doses may be useful for Alzheimer's diseases (where the cholinergic deficit is more severe) it may be overdosing schizophrenia patients.

A number of other compounds have been suggested as possible adjunctive treatments for improving cognitive impairment in schizophrenia, although few trials have been conducted. Galantamine (an AChEI and α4β2 nicotinic receptor allosteric

modulator) has been shown to improve verbal fluency and errors of commission on a continuous performance task when given as an adjunct to risperidone in a 4-week randomized controlled trial (McEvoy and Allen 2003). To our knowledge no other clinical trials have been conducted using galantamine, but given such findings further research is warranted. An unpublished double-blind placebo-controlled trial of 3 weeks monotherapy with xanomeline (an arecoline derivative which acts as an agonist at muscarinic M_1/M_4 receptors) in 20 patients with schizophrenia, has been found to improve cognition relative to placebo (Shekhar et al 2001 In Raedler et al 2007). Therefore, cholinergic agents, particularly those targeting cholinergic receptors, cannot be ruled out as possible treatment options.

Given that glutamatergic dysfunction has been linked to the pathophysiology of schizophrenia (for reviews see Stone et al 2007; Tamminga 1999; Thompson et al 2004), and that glutamatergic mechanisms can modulate cognitive function (for review see Newcomer and Krystal 2001), agents targeting components of the glutamate system have been proposed as possible treatment options (Black 2005; Lynch 2004). A placebo controlled pilot study found that increasing doses of the ampakine CX516 (which has positive modulatory effects at AMPA glutamamteric receptors), administered as an adjunct to clozapine for 4 weeks, had a positive effect on memory and attention in comparison to a placebo group (Goff et al 2001). Despite this, a recent placebo-controlled add-on trial of 4 weeks CX516 treatment failed to show any effect on cognitive performance (Goff et al 2008). Two other glutamatergic agents glycine and $_D$-cycloserine, have also been trialed as possible cognitive enhancers in schizophrenia. Although an initial dose-finding trial in a sample of 9 schizophrenia patients found 2 weeks of 50mg/day of $_D$-cycloserine significantly improved reaction time on a recognition task, double-blind placebo-controlled trials spanning 8, 16 or 24 weeks have not been able to find any effects of glycine or $_D$-cycloserine on cognition (Buchanan et al 2007; Evins et al 2000; Goff et al 2005; Goff et al 1999). A lack of significant findings may be due to the pharmacokinetic profile of these compounds and the inconsistent ways in which they interact with different types of antipsychotic medications (for review see Millan 2005). In summary, these findings overall suggest that an effective treatment for the alleviation of the cognitive deficits in schizophrenia has yet to be discovered.

6.1.3 Estradiol as a Possible Treatment Option for Cognitive Deficits

A recently proposed treatment for cognitive deficits in women with schizophrenia is the female sex hormone E_2. The rationale for this is based partly on the 'estrogen protection' hypothesis of schizophrenia, which proposes that estrogen may have a neuroprotective role for women who are susceptible to developing the illness, given that the risk of developing schizophrenia is higher in men than women before the age of 40 (Rossler et al 2005) and typical age of onset is on average 3-4 years later in women than in men (Angermeyer et al 1989; Hafner 1998; Lindamer et al 1997; Maurer and Hafner 1995). Research also shows that: (1) up until the menopause the course and overall outcome of schizophrenia is generally better for women compared to men (Salem and Kring 1998), (2) psychopathology can worsen when plasma estrogen levels are low (Halari et al 2004; Riecher-Rossler et al 1994; Seeman 1996), and (3) relapse rates are also higher when estrogen levels are low, such as during the menstrual phase (Bergemann et al 2002; Seeman and Lang 1990), and post-partum period (Chang and Renshaw 1986; Kendell et al 1987). More recently, women with schizophrenia who were taking HT were found to have less severe negative symptoms compared to non-users (Lindamer et al 2001).

A small number of studies have investigated the effects of adjunctive ET on psychopathology in women of child-bearing age with schizophrenia. Kulkarni and colleagues (1996) first demonstrated in an open label pilot study that 20µg oral ethinyl E_2 as an adjunct to antipsychotics for 8 weeks enhanced recovery of acute psychosis when compared to women receiving antipsychotics alone. In a subsequent study overall psychopathology improved after 28 days of 100µg/day or 50µg/day of continuous adjunctive transdermal E_2, with the 100µg dose significantly improving all subscales on the Positive and Negative Syndrome Scale (PANSS) (Kulkarni et al 2001). Similarly, Akhondzadeh and colleagues (2003) found improvements in overall PANSS scores as well as positive and general subscales after adding 50µg/day of oral ethinyl E_2 to haloperidol for 8 weeks. Two other researchers have been unable to replicate these findings (Bergemann et al 2005b; Louza et al 2004), although one found a trend for improved positive symptoms ($p= 0.065$) with adjunctive conjugated equine estrogens (CEE) after 4 weeks (Louza et al 2004).

This finding may have reached significance had the duration of treatment been longer, or possibly if the sample size had been larger (> N = 40). Furthermore, it is likely that the type of ET administered (CEE) had an impact on the results, as this type of ET is less potent than E_2. The later study found no effect of combined estrogen-progesterone treatment (EPT), although this sample comprised patients who were generally asymptomatic (minimum 6 weeks remission of symptoms and Brief Psychiatric Rating Scale score <30 prior to inclusion) (Bergemann et al 2005b). Furthermore, as discussed in Chapter 2, the addition of the progestagen may have mitigated the positive effects of the ET. (For more information on the estrogen protection hypothesis of schizophrenia see Hafner 2003; Halbreich and Kahn 2003).

The relationship between circulating (endogenous) estrogen and cognitive function in women with schizophrenia was first examined by Hoff and colleagues (2001). Higher estrogen levels were strongly correlated with better global cognitive function, as well as language, verbal and spatial memory, executive function, and perceptual-motor speed in women with schizophrenia, aged 27-63 (Hoff et al 2001). A later study in women of child-bearing age with schizophrenia found that serum E_2 levels correlated significantly with measures of learning, memory, verbal fluency, information processing speed and executive function, where higher E_2 levels were related to better performance (Ko et al 2006). Furthermore Ko and colleagues (2006) found that 63% of women displayed hypoestrogenemia, and cognitive performance was significantly better in women with high compared to low estrogen levels. To our knowledge, one research group has examined the effects of adjunctive EPT on cognitive performance in 6 post-menopausal women with schizophrenia, with preliminary findings suggesting that verbal memory and visual memory may improve significantly after 6 months treatment (Chua et al 2005; Good et al 1999). However, these findings have yet to be confirmed and have only been published in abstract form and via the Cochrane Database reviewers, thus these results should be viewed with caution. As discussed in Chapter 2, a number of epidemiological and experimental studies have found EPT or ET alone to enhance cognition or to have a protective effect against age-related cognitive decline in healthy postmenopausal women (for reviews see Hogervorst et al 2000; Zec and Trivedi 2002). Despite large inconsistencies within the literature, extensive review and analysis concluded that

HT has a significant positive, modest effects specifically on verbal memory, abstract reasoning and information processing (Hogervorst et al 2000; Zec and Trivedi 2002). Furthermore, research has shown long-term HT to have a protective effect against the development of Alzheimer's Disease (for review see Pinkerton and Henderson 2005).

The effects of neurochemical or biochemical mechanisms associated with ET's effects on cognitive function are yet to be determined. There are a number of possible mechanisms that may play a role and these have been described in Chapter 1. In summary, it is possible that the cognitive effects of estrogen may be linked to neuroprotective mechanisms including: inhibition of oxidative stress (Behl et al 2000), interaction with glia cells (Bruce-Keller et al 2000; Dodel et al 1999; Liu et al 2005), and modulation of neurotrophic factors (Gibbs 1998; Solum and Handa 2002), calcium influx (Wu et al 2005; Zhao et al 2005) and LTP (Foy et al 1999; Kim et al 2002; Kim et al 2006; Teyler et al 1980). In addition, estrogen's effects on cognition could also be mediated by modulation of specific neurotransmitter systems associated with regulation of cognitive function, including dopamine, serotonin and acetylcholine. Incidentally abnormalities in these neurotransmitter systems are linked to the pathophysiology of schizophrenia (for reviews see Raedler et al 2007; van Veelen and Kahn 1999). Animal studies have shown estrogen to have complex effects on the dopaminergic system including evidence for stimulatory and suppressive effects in the striatum (for review see Di Paolo 1994; Lammers et al 1999; Thompson et al 2000). Estradiol treatment has also been linked to increased $5HT_{2A}$ mRNA and receptor density (and decreased $5HT_{1A}$ mRNA) in areas such as the frontal and cingulate cortices of both animals and humans (Birzniece et al 2001; Kugaya et al 2003; Osterlund et al 1999; Summer and Fink 1995). Research has also found co-localisation of estrogen receptors on cholinergic neurons (Miettinen et al 2002; Shughrue et al 2000; Toran-Allerand et al 1992), increased choline acetyltransferase activity in the basal forebrain after ET (Gibbs et al 1994; Luine 1985; McMillan et al 1996), and a protective effect of ET on cognitive deficits induced by the muscarinic antagonist scopolamine (Dohanich et al 1994; Fader et al 1998; Gibbs 1999; Gibbs et al 1998). Human studies are less consistent as E_2 treatment has been found to attenuate the scopolamine-induced impairments on

attention and verbal memory tasks for postmenopausal women (Dumas et al 2006; Dumas et al 2008), but not in our sample of young healthy young women (see Chapter 5). It may be that E_2's effects on the cholinergic system and cognition are age-dependent, as the length of HT in women has been positively correlated with vesicular acetylcholine transporter binding indexes in area such as the frontal and cingulate cortices (Smith et al 2001), brain regions essential for cognitive processes such as attention, learning and memory.

These findings, along with the evidence supporting estrogen's neuroprotective effects in schizophrenia, and superior performance on certain cognitive tasks in postmenopausal women taking estrogen, provides strong grounds for examining the effects of estrogen in the treatment of cognitive impairment in women of child-bearing age with schizophrenia. It is paramount that this relationship be explored in young and middle-aged women with schizophrenia, given that the majority of women with this mental illness experience cognitive deficits from the initial onset of psychosis which typically occurs during the late 20s (Hafner 2003; Salokangas et al 2003). Thus, the current study aimed to examine the neurocognitive effects of one month of 100μg/day of adjunctive E_2 treatment in women of child-bearing age with schizophrenia.

6.2 Methods and Materials

6.2.1 Participants

Women were of child-bearing age (M = 34.7, SD = 7.9) with a DSM-IV diagnosis of schizophrenia, or schizoaffective disorder (non-manic phase), as determined by a psychiatrist. Participants were taking part in a larger study investigating the effects of estrogen on psychopathology, findings of which will be presented elsewhere (Kulkarni et al 2008). Women were excluded if they were pre-menarche, post- or peri-menopausal, pregnant or lactating, taking oral contraceptives or synthetic steroids, had abnormalities in the hypothalamo-pituitary gonadal axis, thyroid dysfunction, central nervous system tumors, or had other serious medical conditions which would contraindicate estrogen use. Women were excluded if their diagnosis

was related to head injury, postpartum psychosis or illicit drug use, or if they had an intellectual disability or had abused drugs/alcohol within the last 6 months. Women were recruited from both inpatient wards and outpatient clinics affiliated with either the Dandenong Area Mental Health Services or Bayside Health Services. This study was approved by the Alfred Human Research Ethics Committee of the Alfred Hospital and The Sourthern Health Care Network, Dandenong Hospital Ethics Committee. All participants gave written informed consent. The majority of participants were approached after consultation with their treating psychiatrist and after a full review of psychiatric and medical history. A small number of participants contacted the research team after hearing about the trial from another source. Participants were given a plain language statement, which they were to read or have read to them, and were encouraged to discuss the trial and their decision with a friend or family member. It was emphasized that participation was completely voluntary and that participants were free to withdraw at any time. Assessment of an individual's ability to give informed consent involved a brief discussion, during which participants were required to explain their understanding and basic procedure of the study. If in the event the individual did not understand, or if the researcher and primary investigators were unsure as to their ability to give informed consent, they were consequently excluded from the study.

6.2.2 Study Design

This study employed a double-blind placebo-controlled between groups design. Participants were randomly assigned to either receive 100μg/day of adjunctive transdermal E_2 (Dermestril 8mg skin patch, Mayne Pharma (USA) Inc.) (n = 27) or transdermal placebo (n = 23) for 4 weeks. The dose and duration of treatment were chosen based on previous findings of significant positive effects observed on psychopathology (Kulkarni et al., 2001). Participants were instructed to change the patch every 3-4 days, thus E_2 administration was continuous. Participants were given detailed written and verbal instructions on how to apply the patches (see Appendix H), and on request were aided or given a demonstration on how the patch was to be applied. Compliance was monitored on a weekly basis either in person or via the phone. All participants remained on antipsychotic medication as prescribed

by their treating psychiatrist, most of which were of the atypical type however a small number were taking typical antipsychotics which included: Haloperidol, Zuclopenthixol, Clopixol, Flupenthixol, Chlorpromazine hydrochloride and Fluphenazine (see results section Table 10). Women started the trial as soon as consent was obtained, thus some women began treatment while in the follicular phase (days 1-14) of their cycle and others began when in the luteal phase (days 14-28). Cycle phase was determined via self-report and via the baseline hormone values: follicular = E_2 143-694 pmol/l; progesterone 0.6-2.6 nmol/l; LH 1-26 IU/l; and FSH 2.5-10.2 IU/L; luteal = E_2 176-1134 pmol/l; progesterone 13.2-75.2 nmol/l; LH 1-27 IU/l; and FSH 1.5-9.1 IU/L. The cognitive battery (see below) and menstrual cycle questionnaire (see Appendix G) was administered at baseline and 28 days post treatment. Psychopathology and physical abnormalities were monitored at baseline and on a weekly basis using the PANSS (Kay et al 1987), Adverse Symptoms Checklist, Abnormal Involuntary Movements Scale (AIMS) (Guy 1976), Simpson Angus (Simpson and Angus 1970), and general health questionnaires. Note that, only data relevant to the aims of the current paper were analyzed. Venous blood samples were collected at baseline and at weekly intervals to measure plasma E_2, LH, FSH, prolactin, progesterone and testosterone. Blood samples were centrifuged at 4000rpm and frozen at -4 degrees C until assayed. Analysis of E_2, progesterone and testosterone was conducted on a Roche E170 instrument using an electrochemiluminesience assay, and samples were stored frozen at -20 degrees Celsius until analysis was completed. Prolactin, LH and FSH was analysed on an Abbott Archicentre instrument using a chemiluminescent immunoassay, and analysis was completed in real time.

6.2.3 Cognitive Measures

The cognitive battery was chosen based on the primary cognitive domains affected in schizophrenia, while considering the cognitive processes that have previously been sensitive to ET.

6.2.3.1 Declarative Verbal Memory & Learning

California Verbal Learning Test (CVLT): (Delis et al 1987) was used as a measure of learning and short and long term memory (Woods et al 2006). Participants were verbally presented a list of 16 items (list A) in the form of a shopping list (eg. 'grapes', 'vest'). The 16 items belonged to four different categories; fruits, spices/herbs, tools and clothing, which was for the purpose of aiding in the learning and recall of the items. Participants were instructed to say back as many items as possible, in any order, immediately after the list was read. This procedure was repeated 5 times before participants were presented with a second list (list B) of shopping items as an interference trial, and again participants were asked to recall as many words as possible from this list. Immediately after participants were done recalling items from the second list they were asked to recall from memory, as many items from the first shopping list. After a 20 minute delay period participants once again performed free recall of the first shopping list. The outcome measures used for the analysis were total number of item recalled from trials 1-5 of list A, and the long-delay free recall trial of list A.

6.2.3.2 Verbal Fluency

Controlled Oral Word Association Test (COWAT): is a measure of Verbal fluency, which can be described as one's ability to generate words related to a given category in a limited amount of time (Benton and Hamsher 1976). Participants were asked to verbally produce as many words that began with a particular letter (e.g. F), in 1 min. This was repeated using the letters A and S. Participants were also instructed to try not to produce proper nouns, numbers, repeat words, or repeat the same word with a different suffix. The outcome measures for verbal fluency were the total number of correct words produced across the three trials and the number of errors produced across the three trials.

6.2.3.3 Visual Memory

Visual Reproduction subscale of the Wechsler Memory Scale – Third Edition (WMS-III): was administered to test immediate and delayed visual memory (Wechsler 1997). Participants were shown a series of line drawings (5 in total), which were displayed for 10 seconds each. After each design was displayed participants were

asked to draw the design as accurately as they could remember, taking as much time as needed. After 25 minutes participants were asked to again recall and reproduce as many of the designs as possible. This task required not only visual memory, encoding of visual stimuli and retrieval but also a degree of visuospatial skills. The outcome measures used for the analysis were the total scores obtained for immediate recall and the total score obtained for the delayed recall trial.

6.2.3.4 Working Memory

Digit Span Backwards (subtest from the WMS-III): was administered as a measure of working memory (Wechsler 1997). Digit span backwards requires participants to recall a list of numbers verbally produced by the researcher and then repeat them back verbally in the reverse order. The task load begins with two numbers and increases every two trials, providing the participant gets at least one of the trials in each block correct. There is a maximum of 14 trials with the longest string being a total of eight numbers. This task is thought to involve elements of working memory given that the numbers must be held 'on line' and then manipulated to be rearranged into a representation different to that initially held in immediate memory (Lezak 1995). The outcome measure used for the analysis was the total score from all trials.

6.2.3.5 Cognitive Flexibility

Stroop Color and Word Test: The traditional paper and pencil version of the Stroop Color and Word Test (Golden 1978; Stroop 1935) was administered as a measure of executive function, response inhibition and attentional control, specifically requiring the ability to flexibly adjust attention (MacLeod 1991). This task had three separate trials, each lasting 45 seconds. Participants were instructed to read aloud, as fast as possible, a series of; names of colors (green, blue, red) written in black ink, blocks of actual colors (appearing as 'XXXX') and colors written as the word of an incongruent color (such as the word 'green' written in red ink). This third trial tests the speed of recognition and response when two perceptual processes conflict. Because individuals have a learned automatic response to read words rather than the colors they are written in, a conflict arises when the innate response must be inhibited and a new set of rules applied, thus an interference occurs. The outcome

measure used in the current study was the interference score, calculated using the total number of words read (within the 45 sec) for each of the three trials.

6.2.3.6 Attention

Digit Span Forwards (subtest from the WMS-III): was administered as a measure of attention and involves the immediate verbal recall of numbers (Wechsler 1997). As in the Digit Span backwards trial a series of numbers are verbally presented to the participant who is asked to repeat the numbers back in the exact same order. Initially the first two trials begin with only 2 numbers each and increase by 1 number for every two trails leading to a maximum of 9 numbers (16 trials). Again, like Digit Span backwards participants must correctly repeat at least one of the trials in each block in order to move onto the more difficult trial. The Digit Span forwards measure is thought to be more closely a reflection of the efficiency of attention (i.e freedom from distractibility) than what is commonly though of as memory (Lezak et al 2004). The outcome measure used for the analysis was the total score from all trials.

6.2.3.7 Psychomotor Function and Information Processing

Trail Making Test (TMT): was included as a measure of psychomotor speed and information processing (Reitan 1992). In Trail A participants were given an A4 sheet of paper which contained 25 numbered circles dispersed in various locations on the page. Participants were asked to join the circles in numerical order as fast as possible without lifting the pencil off the page. Trail B was similar to Trail A however half of the circles were numbered 1-13 and the other half were labeled A-L and positioned in various locations on the page. Trail B not only required attention but also perceptual processing as participants were asked to connect the circles in numerical and alphabetical order by alternating between the two, such as 1-A-2-B-3-C etc. If participants made an error they were notified of this and instructed to go back to the previous circle. The outcome measures from this test were the total time (sec) taken to complete Trail A and the total time taken to complete Trail B.

During the delay periods of the CVLT and Visual Reproduction test participants performed the other tasks in the cognitive battery.

6.2.4 Statistical Analysis

Data was analyzed using SPSS v15 (SPSS for Windows, version 15, SPSS Inc., Chicago, IL, USA). To determine whether there were any significant differences between the two treatment groups at baseline, t-tests and Pearson's Chi-Square Tests were performed on demographic variables. Separate mixed ANOVAs (with Huynh-Feldt adjustments where appropriate) were conducted on the following cognitive measures (which pertain to the 7 chosen cognitive domains): CVLT total words recalled trials 1-5 List A, CVLT long-delay free recall List A (declarative verbal memory and learning), COWAT total words, COWAT total errors (verbal fluency), Visual Reproduction immediate & delayed (visual memory), Digit Span backwards (working memory), Stroop Interference score (cognitive flexibility/executive function), Digit Span forwards (Attention) and TMT A and B (psychomotor function/information processing). Given the difficulty with recruiting from this clinical population the menstrual cycle phase that participants were in at the initiation of the trial varied, thus Menstrual Cycle Phase was included as a factor for all mixed ANOVAs. Mean scores on these cognitive measures were the Dependent Variables, and the between-groups factors 'Treatment Group' (estradiol/placebo) and 'Menstrual Cycle Phase' (follicular/luteal), as well as the within-group factor 'Time' (baseline/post-treatment) were the Independent Variables. Post hoc analyses were employed for significant effects/interactions with bonferroni corrections (corrected p-values reported). This method of analysis was chosen based on previous research which used this method to investigate the effects of antipsychotics on cognition in schizophrenia (Daban et al 2005; Hori et al 2006; Sharma et al 2003; Sumiyoshi et al 2003), and after taking into consideration the wide range of cognitive domains that have been found effected by ET in the past (see Chapter 2 for review).

In order to reduce skewness and meet the normality assumptions, outliers (identified using the boxplot inter-quartile range function in SPSS) that were deemed actual measures of performance and therefore part of the target population, were transformed in order to reduce the skewness of the group's mean value. This was achieved by assigning a value of one unit more extreme than the next most extreme value in that group's population, as suggested by Tabachnick and Fidell (2001).

Overall 1.36% of the total values included in the analysis of cognitive data were transformed using this method. Analyses involving amended values were re-run using original change scores, to ensure that transformation of outliers did not substantially impact on the results.

We further investigated the effects of pre-treatment endogenous hormone levels on cognition using Pearson's correlation coefficients. Baseline scores on each cognitive measure was correlated with baseline E_2 (pmol/L) level. Similarly, baseline cognitive scores were also correlated with baseline progesterone (nmol/L) level for each cognitive measure. This was to test the reliability of this statistical procedure for examining the relationship between endogenous ovarian hormones and cognitive performance, and whether findings would be consistent with Hoff and colleagues (2001).

6.3 Results

6.3.1 Participants

Fifty participants were enrolled in the current study (estrogen n = 27, placebo n = 23) and all returned for the day 28 assessment. However, 1 participant did not use the patches for the entire 4 weeks, 3 were unable to perform the cognitive tests due to their mental state at the time of assessment, thus these participants were excluded from the primary analyses (see Figure 12). As mentioned in section 6.2.1 of the methodology, participants were taking part in a larger study when they enrolled to also take part in the current protocol, thus the uneven number of participants per group is due to randomization of treatment, which was in accordance with the larger study.

The two groups were very similar in clinical characteristics as there were no significant differences found for any of the demographic variables (see Table 10). The majority of women were diagnosed with schizophrenia and were moderate-severely ill at the time of inclusion, having suffered from their illness for approximately 11-12 years. 81% and 78% of women from the estrogen and placebo

groups respectively, had previously been on the contraceptive pill, however all participants had been estrogen-free for a minimum of one month. It is important to note that we did not exclude smokers, given that inclusion criteria was already quite specific and that a larger percentage of individuals with schizophrenia compared to the normal population, are cigarette smokers (Hughes et al 1986; Kelly and McCreadie 1999). The number of moderately heavy smokers in the current sample was quite large, however there were relatively equal numbers in the E_2 (67%) and placebo (57%) groups. All participants were receiving antipsychotic medication prior to inclusion, and although the medication type varied the majority of women (84%) were taking atypical antipsychotics (see Table 10). There was no significant group difference in the average risperidone equivalent dosage, calculated using the computer program designed by Tim Lambert (1999). Furthermore, independent samples t-tests revealed no significant differences in baseline cognitive performance between the two groups on any cognitive measure.

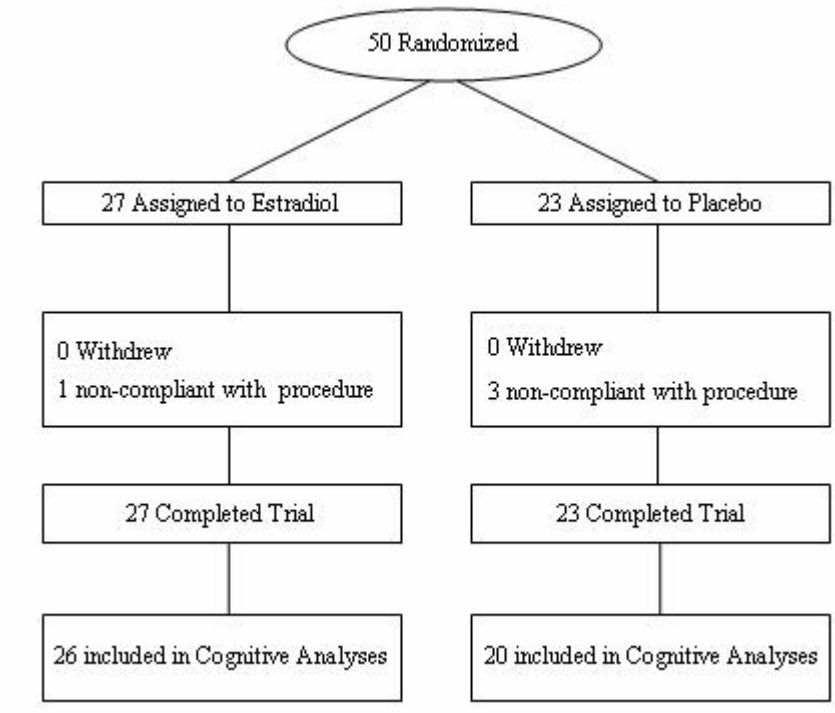

Figure 12. Flow Diagram of Study Population

Table 10. Demographic Characteristics

	Estradiol Group (n = 27)	Placebo Group (n = 23)	T-test/χ^2 p value
M age (SD)	34.6 (8.9)	34.8 (6.8)	.93
M length of illness (yrs) (SD)	12.1 (9.1)	11.2 (6.9)	.74
M number of hospital admissions (SD)	8.9 (12.6)	8.6 (8.1)	.92
Severity of illness at baseline (*M* PANSS total score)	77.8 (16.6)	75.6 (12.8)	.61
M Risperidone equivalent dose[a] (mg)	13.01 (18.0)	11.26 (19.5)	.74
Medication type (n)			.41
Atypicals: Risperidone	5	3	
Olanzapine	6	3	
Clozapine	11	7	
Quetiapine	3	2	
Amisulpride	0	2	
Typicals: Thioxanthene type	1	3	
Phenothiazine type	1	2	
Butyrophenone type	0	1	
N taking antidepressants	8	5	.45
N taking anxiolytics	6	6	.75
Diagnosis			.86
Schizophrenia	23	20	
Schizoaffective Disorder	4	3	
Menstrual cycle phase at baseline			.20
N Follicular	11	14	
N Luteal	15	9	
M E_2 level at baseline (pMol/L)			
Follicular[b]	206.2 (178.6)	252.2 (207.8)	.56
Luteal[c]	375.7 (211.8)	287.6 (108.8)	.20
N with regular menstruation	22	18	.78
N with previous history of ET	23	18	.75
M years duration of ET (SD)	2.7 (2.4)	2.3 (2.8)	.64
N current smokers	18	13	.73
M cigarettes/day (SD)	26.3 (17.5)	24.9 (15.1)	.81

Note: PANSS- Positive and Negative Syndrome Scale, ET- Estrogen Treatment, *M*- mean, *N* - number of participants. [b]Normal follicular E_2 levels range from 143-694. [c]Normal luteal E_2 levels range from 176-1134.

6.3.2 Estradiol as an Adjunct to Antipsychotics

6.3.2.1 Cognitive Effects

The analysis of the Visual Memory domain revealed a significant interaction between Treatment Group and Time for the immediate recall visual memory measure ($F(1,40) = 4.47$, $p =<.05$, partial $\eta^2 = .10$) (see Figure 13). Post hoc repeated measures ANOVAs for the two treatment groups showed that the E_2 group displayed no real change in performance over time (p= NS), while the placebo group had

improved significantly on the immediate recall task ($F(1,18) = 13.58$, $p<.01$, partial $\eta^2 = .43$). The analysis also found a significant main effect of Time for both immediate ($F(1,40) = 15.01$, $p<.001$, partial $\eta^2 = .27$) and delayed recall ($F(1,39) = 19.15$, $p<.001$, partial $\eta^2 = .33$), where participants overall scored higher on day 28 of treatment compared to baseline. There were no other main effects or interactions found for the visual memory domain (see Table 11 for means and standard deviations of cognitive measures).

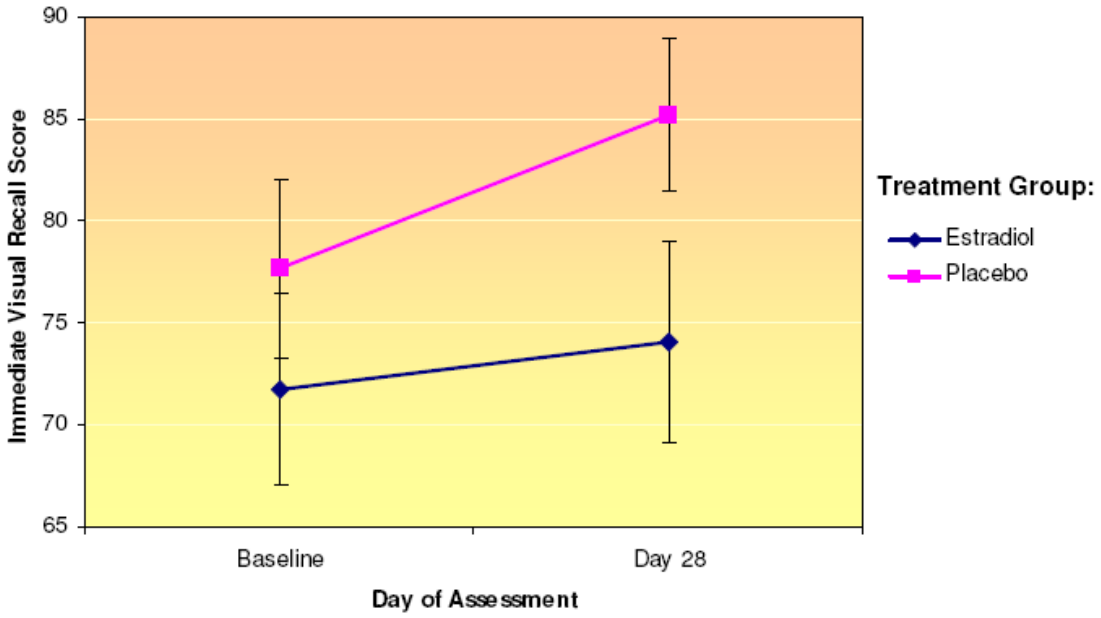

Figure 13. Effects of one month adjunctive E_2 treatment on visual memory in women with schizophrenia. Means ± SEM displayed for the immediate recall of WMS III Visual Reproduction subtest. The placebo group improved significantly ($p<0.05$) over time while performance by the estradiol group remained stable.

A significant interaction was found between Time, Treatment Group and Menstrual Cycle Phase for the measure of attention (digit span forwards; $F(1,39) = 5.32$, $p<.05$, partial $\eta^2 = .12$) (see Figure 14). Post hoc mixed ANOVAs were conducted separately for women in the two different menstrual cycle phases. There were no main effects or differences between groups in the women who initiated the trial in the follicular phase, however there was a significant interaction between Time and Treatment Group for women in the luteal phase ($F(1,19) = 7.94$, $p<.05$, partial $\eta^2 = .30$). Further post hoc analyses for the E_2 (luteal phase only) and placebo (luteal phase only) groups separately, did not produce any significant effects.

Table 11. Means and Standard Deviations of Cognitive Measures at Baseline and Post-Treatment

	Estradiol Group (n = 26)		Placebo Group (n = 20)		P value
	Baseline	Day 28	Baseline	Day 28	Time x Group
Cognitive Measure	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Declarative Verbal Learning & Memory					
CVLT:					
List A total (Trial 1-5)	42.72 (13.26)	46.68 (14.65)	42.85 (13.48)	45.05 (13.96)	.499
Long-delay free recall	8.92 (4.16)	9.63 (3.94)	8.78 (3.59)	9.50 (3.54)	.816
Verbal Fluency					
COWAT:					
Total words	35.04 (14.36)	38.16 (14.35)	34.89 (12.32)	37.32 (12.74)	.887
Total errors	3.04 (3.13)	3.24 (3.68)	2.37 (2.43)	3.05 (3.44)	.610
Visual Memory					
Visual Reproduction:					
Immediate recall	71.76 (23.50)	74.08 (24.53)	77.63 (19.05)	85.21 (16.40)	.041*
Delayed recall	51.24 (27.06)	61.08 (27.53)	61.56 (32.82)	69.06 (28.14)	.717
Working Memory					
Digit Span:					
Backwards	5.04 (2.14)	5.33 (1.69)	5.53 (1.81)	5.47 (2.27)	.495
Cognitive Flexibility					
STROOP:					
Interference score	-0.62 (7.31)	0.17 (6.59)	-3.49 (8.64)	-1.15 (7.86)	.292
Attention					
Digit Span:					
Forwards	9.42 (1.89)	9.88 (2.49)	10.26 (2.64)	10.00 (3.07)	.114
Psychomotor Function & Information Processing					
Trails Making Test:					
Trail A (sec)	32.88 (11.04)	35.96 (15.02)	32.68 (9.67)	28.42 (7.71)	.007**
Trail B (sec)	97.48 (46.32)	85.22 (30.35)	91.56 (37.93)	74.39 (27.97)	.485

Note: SD- standard deviation, RM – repeated measures, COWAT – Controlled Oral Word Association Test, CVLT – California Verbal Learning Test, * significant at p<.05 level, **significant at p<.01 level.

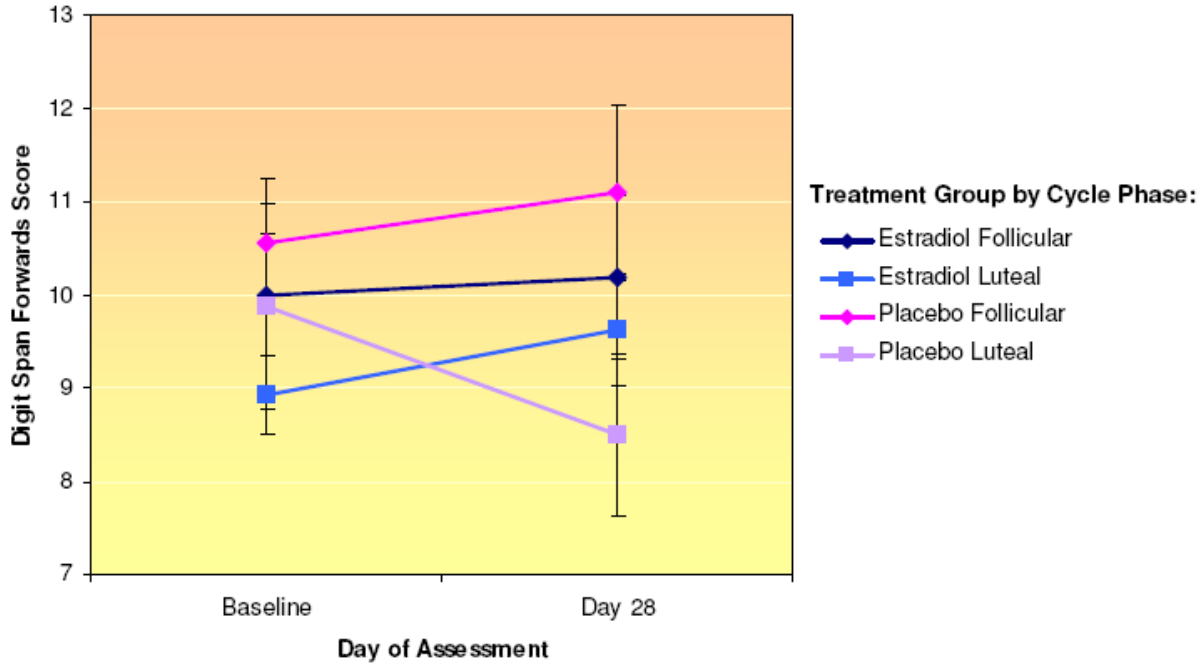

Figure 14. Effects of one month adjunctive E_2 treatment on attention in women with schizophrenia. Means ± SEM displayed for the forwards condition of the Digit Span subtest of the WMS III. For women in the Luteal phase only, change in performance differed significantly between the estradiol and placebo groups ($p<.05$).

Analysis of Psychomotor Function and Information Processing revealed a significant interaction between Time and Treatment Group for the TMT A measure ($F(1,40) = 8.22$, $p<.01$, partial $\eta^2 = .17$) (see figure 15). Separate post hoc ANOVAs showed that there was no significant change in performance for the E_2 group (p= NS), while the placebo group improved significantly over the one month period ($F(1,18) = 9.45$, $p<.05$, partial $\eta^2 = .34$). However, caution should be taken when interpreting this result as analysis on untransformed TMT A data did not reveal any significant effects. There was a significant main effect of Time for the Trail B measure ($F(1,37) = 10.62$, $p<.01$, partial $\eta^2 = .22$), where all participants in general improved in performance from baseline.

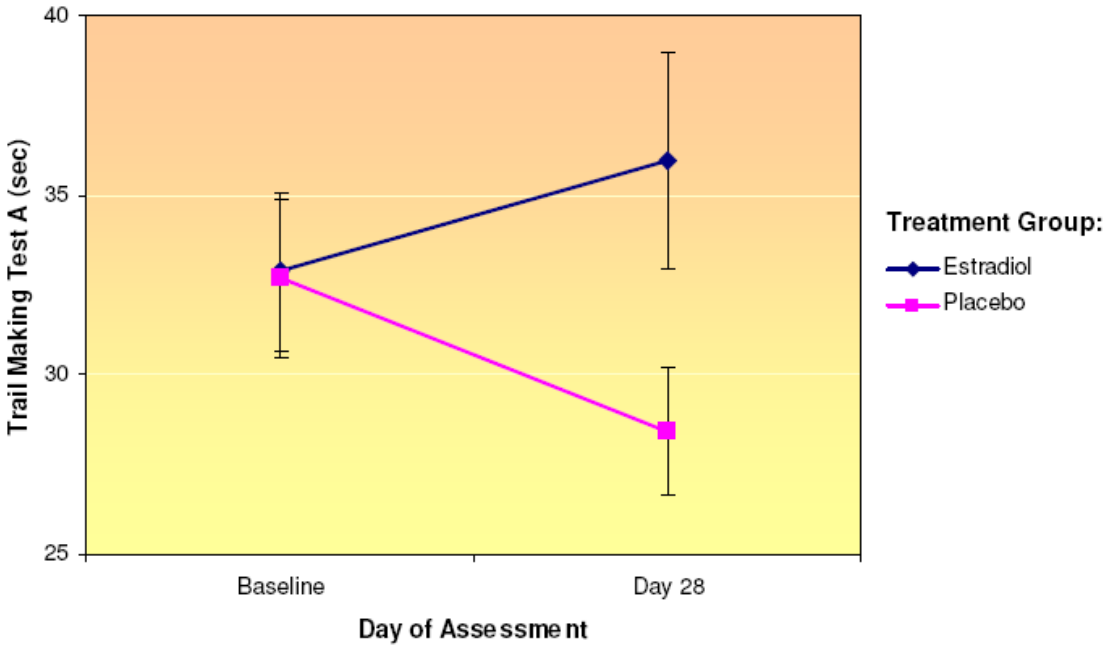

Figure 15. Effects of one month adjunctive E_2 treatment on psychomotor function and information processing in women with schizophrenia. Means ± SEM displayed for Trail A of the TMT. Lower scores represent better performance. The placebo group improved significantly over time ($p<0.05$), while there was no significant change in performance of the E_2 group.

There were no significant interactions found for any of the other cognitive measures. Lastly, there were significant main effects of Time for both List A total trials 1-5 ($F(1,41) = 5.21$, $P<.05$, partial $\eta^2 = .11$) and long-delay free recall ($F(1,38) = 8.09$, $p<.01$, partial $\eta^2 = .18$) of the verbal memory and learning domain, as well as COWAT total words ($F(1,40) = 5.72$, $p<.05$, partial $\eta^2 = .13$) of the verbal fluency domain, where again participants generally improved over time. There were no significant main effects found for working memory or cognitive flexibility (see Table 11). Results did not change when 'age' was included in the analysis as a covariate.

6.3.2.2 Effects on Psychopathology

Psychopathology was analyzed using three separate 2 (Treatment Group) x 2 (Time) mixed ANOVAs for the positive, negative and general subscales of the PANSS. A significant interaction between Treatment Group and Time was found for the positive symptoms subscale ($F(1,45) = 5.58$, $p= <.05$, partial $\eta^2 =.11$). Separate post hoc ANOVAs showed that the E_2 group improved significantly on the rating of positive

symptoms (baseline *M* (SD) = 21.15 (6.74), day 28 *M* (SD) = 16.58 (5.36); F(1,26) = 24.30, p<.001, partial η^2 = .48), while the placebo group showed no change over time (baseline *M* (SD) = 20.74 (5.85), day d8 *M* (SD) = 20.26 (8.45); p = NS). There was no significant difference between the groups on the negative or general rating scales of the PANSS. It should be noted that the effects of estrogen treatment on psychopathology have been investigated at greater lengths in the larger study from which this sample was derived, therefore discussions will focus on findings relevant to the aims of the current thesis.

6.3.3 Estradiol Levels and Cognition

Unexpectedly the mean E_2 level (pmol/L) at day 28 did not change significantly from baseline for the estrogen group (baseline M = 301.12, SD = 212.05; day 28 M = 304.60, SD = 188.09), nor was the average group level significantly different from the placebo group (baseline M = 266.04, SD = 173.60; day 28 = 293.61, SD = 287.18) at either baseline or day 28 according to independent samples t-tests. Given these findings and the fact that participants started the study during different phases of the menstrual cycle, separate t-tests were then run for those who initiated treatment in the 'follicular phase' and those who initiated treatment in the 'luteal phase' of the menstrual cycle. There were no significant differences found between the two groups at any time point for either follicular or luteal participants, including at day 7, 14 and 21 of treatment (see figure 16 for plasma E_2 levels across the 28-day treatment period).

We further investigated the possible effects of endogenous estrogen levels on cognition prior to treatment allocation, in order to test the reliability of this method of analysis in assessing the relationship between E_2 and cognition. The results revealed no significant baseline correlations between E_2 level, or progesterone level, and any of the cognitive measures.

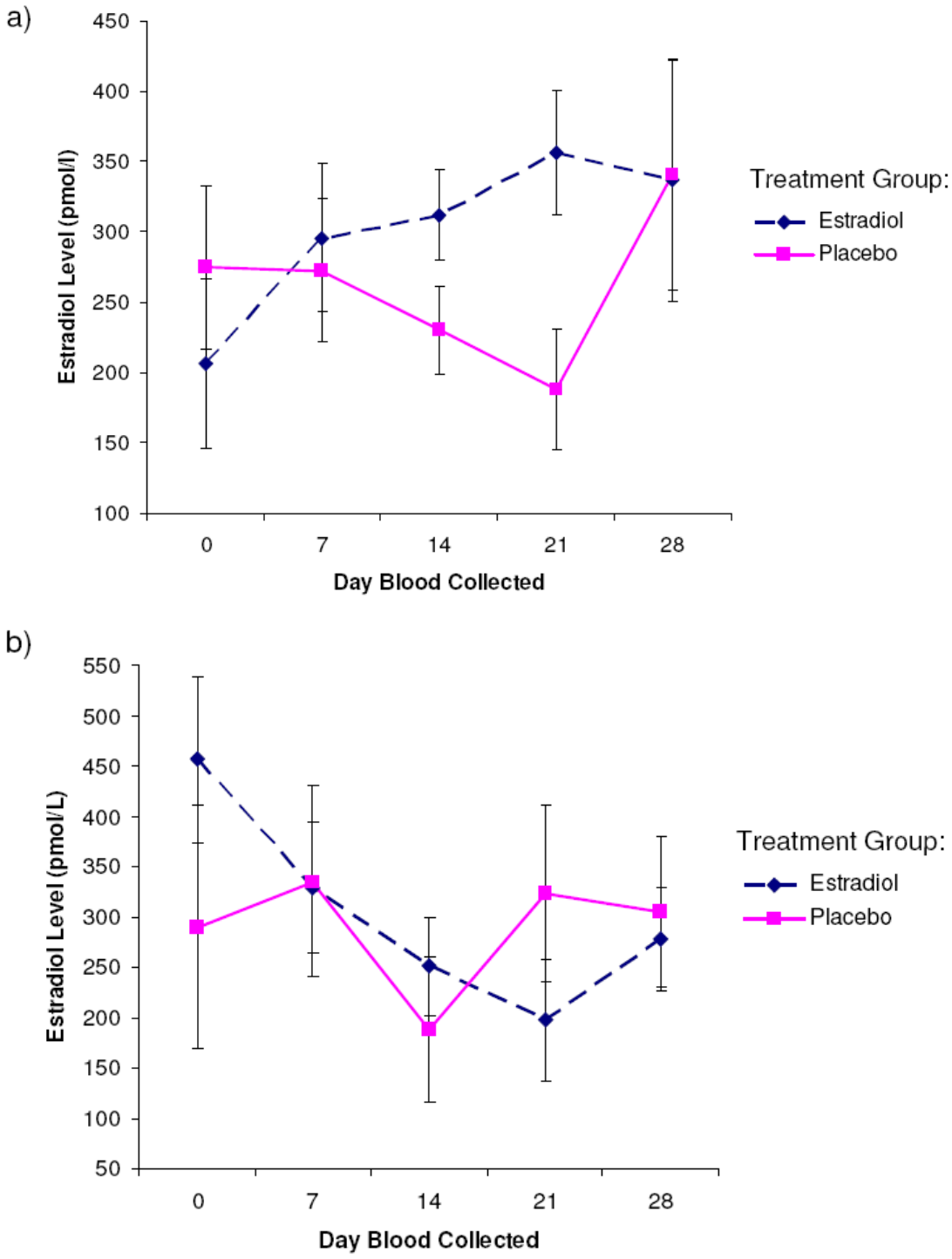

Figure 16. Estradiol level at weekly intervals during the one month treatment period for women with schizophrenia. Mean ± SEM displayed for a) participants who started the study during the follicular phase (N = 25); and b) participants who started the study during the luteal phase of the menstrual cycle (N = 24). There were no significant differences in plasma E_2 levels between the two groups.

6.4 Discussion

This study is the first double-blind placebo-controlled study to investigate the cognitive effects of adjunctive E_2 treatment in a sample of women of child-bearing age with schizophrenia. The findings suggest that the addition of 100µg of E_2 (to antipsychotic medication) for one month had no beneficial effect on cognitive performance. These findings are partially in agreement with our findings in healthy young women reported in Chapter 5 where the majority of cognitive processes were unaffected by E_2 treatment. Given that there are no previous published studies of this nature in patients with schizophrenia, it is difficult to compare the current findings to that of past research. Unexpectedly, in the instances where an interaction was found between the two treatment groups (as with visual memory and psychomotor function/ information processing), it was consequently the placebo group that improved in performance rather than the E_2 group. The current findings are in contrast to the data collected by Good and colleagues (Good et al 1999), and although their analysis was preliminary, findings suggested ET may improve visual memory for post-menopausal women with schizophrenia (Chua et al 2005). Similarly, the current findings also do not support experimental research in healthy post-menopausal women which has shown short-term ET to improve visual memory (Duka et al 2000; Resnick et al 1998; Resnick et al 1997) and speed of information processing (Sherwin 1988). As outlined in chapters 2 and 5, research in this area is inconsistent and numerous placebo-controlled experimental studies have conversely failed to find an effect of ET on visual memory and information processing (Almeida et al 2006; Dumas et al 2006; Dumas et al 2008; Goebel et al 1995; Janowsky et al 2000; Joffe et al 2006; LeBlanc et al 2007; Pefanco et al 2007; Phillips and Sherwin 1992; Polo-Kantola et al 1998; Rauramo et al 1975; Shaywitz et al 1999; Wolf et al 1999; Yaffe et al 2006).

The current findings are unlikely to be related to a lack of efficacy of E_2 dosage, as positive symptoms improved significantly for the women in the estrogen group compared to the placebo group, which is in line with previous research (Kulkarni et al 1996; Kulkarni et al 2001). This finding itself supports the general consensus that cognitive deficits are not simply a bi-product of positive symptoms, and do in fact

need to be addressed as an independent symptom dimension in the treatment of schizophrenia. It may be that a 100µg dose of E_2 is effective for improving schizophrenia psychopathology, but that the threshold for improving cognitive deficits may require a higher or possibly a lower E_2 dose. In addition, it appears that endogenous fluctuations in ovarian hormones had little impact on cognitive performance or on the way in which E_2 treatment affected cognition. This is based on the finding that menstrual cycle phase was not a significant determining factor in all analyses, with the exception of the attention domain (although post hoc analyses were non-significant). While the current findings do not support a number of experimental studies in healthy postmenopausal women which show improvements in various cognitive domains following ET (Asthana et al 2001; Duka et al 2000; Fedor-Freybergh 1977; Hogervorst et al 1999; Joffe et al 2006; Krug et al 2006; Linzmayer et al 2001a; Linzmayer et al 2001b; Phillips and Sherwin 1992; Saletu 2003; Shaywitz et al 2003), findings are in line with other experimental studies in postmenopausal women which have not been able to find any effect of estrogen on cognitive functions (Almeida et al 2006; Binder et al 2001; Ditkoff et al 1991; Goebel et al 1995; Janowsky et al 2000; Kurt et al 2006; Leblanc et al 2007; Yaffe et al 2006). Together these studies further highlight the complexity and variability in ET effects on cognition.

Previously, strong positive correlations between E_2 levels and compounded scores of verbal memory, executive function, language, concentration/speed and spatial memory have been observed in patients with schizophrenia (Hoff et al 2001). In the current study Pearson's correlations of baseline circulating plasma E_2 levels with cognitive performance revealed no linear relationships between E_2 and cognitive performance. However, as mentioned previously the use of correlation analyses is an unreliable method for assessing the effects of E_2 on cognition and has previously yielded inconsistent mixed results (for review see Chapter 2). In addition, recent evidence suggests circulating plasma levels of E_2 do not accurately reflect E_2 levels in the brain, given that certain brain regions produce their own estrogens locally (Simpson 2003). Nevertheless it is worth exploring possible methodological differences between the current study and that by Hoff and colleagues (2001) which may explain the discrepant findings. For example, Hoff and colleagues (2001) tested

women aged 27 to 63 (M = 40.2), while women in the current study were all of child-bearing age (M = 34.7). Thus, the younger women in their study who had not experienced menopause or age-related cognitive decline would be expected to have higher E_2 levels and perform better than the older peri/postmenopausal women, increasing the likelihood of finding positive correlations. Although Hoff and colleagues (2001) found no correlation between estrogen level and age, there may have been a confounding effect of age on cognition, especially seeing as there was no report of older women being screened for dementia. Furthermore, the analysis employed by Hoff and colleagues (2001) was somewhat different to ours, as they used an average serum E_2 level, collected weekly over a 4 week period. Hence, the nature of the relationship Hoff and colleagues (2001) examined was different to that of the correlations performed in the current study. Hoff and colleagues (2001) investigated the effect of general E_2 level on cognitive function which is possibly related to neuroprotective mechanisms over a longer period of time. The correlations in the current study analyzed state E_2 levels at the time of cognitive testing, which may be more likely related to an interaction with neurotransmitter systems involved in the cognitive processes being tested. The findings of the current study do however support those of Thompson and colleagues (2000) who also investigated women with schizophrenia and used the same method of analysis, which revealed no correlations between cognitive performance and endogenous E_2 levels collected on the same day as cognitive testing.

Results are not likely to be related to sample size as there were enough participants to detect moderate-large effects with 80% power. Previous experimental research in women has detected small and large effects of ET on cognition (ranging from .22 - .96) (Zec and Trivedi 2002), suggesting that if estrogen had a similar effect on cognition in women with schizophrenia then our sample size was more than adequate. The majority of effect sizes detected in the current study were very small (between .00 and .12), suggesting that, according to (Cohen 1992), we would need a sample size of at least 393 for the statistics to be significant, yet an effect so small would not be of any practical use or physiologically relevant. It may be that the duration of treatment was insufficient to elicit an effect on cognitive performance, as improvements have typically been observed after at least 8 weeks of E_2 treatment in

healthy postmenopausal women (Phillips and Sherwin 1992). Although two studies have found significant improvements on verbal memory after just 2 weeks (Wolf et al 1999), and on memory and spatial skills after only 3 weeks of ET (Duka et al 2000), the authors of these studies failed to find significant effects on a number of other cognitive tasks administered, including measures of learning, executive function, inhibition and planning skills, spatial memory and spatial skills.

Although the current study did not include a healthy control group, it is clear from previous research that women in both groups were performing below normal levels, as scores were comparable to those reported by Hoff and colleagues (1998) in which women with schizophrenia, compared to healthy controls, performed significantly worse on many of the same cognitive measures as those used in the current study. Similarly, women in the current study also had markedly lower E_2 levels in comparison to women in the general population, based on the previously reported mean E_2 levels of healthy cycling women to be around 520-560 pmol/L (Huber et al 2004; Huber et al 2001; Riecher-Rossler et al 1994). Mean E_2 levels for women in the current study (see Demographics, Table 10) were comparable to those of Thompson and colleagues (2000) who found the mean estrogen level of psychotic patients to be 241.8 pmol/L during follicular phase and 360.5 pmol/L during the luteal phase. In addition, a number of women in the current study had grossly abnormally low E_2 levels (24% had levels < 150 pmol/L at baseline; see Appendix M for raw hormone data), equivalent to that of post-menopausal women.

The low E_2 levels of women in the estrogen group may be due to varying rates of E_2 metabolism or interactions with other medications (Yonkers et al 1992). Specifically, drugs such as carbamazapine, phenobarbital and rifampin induce the enzyme that primarily metabolizes E_2 (CYP3A4), which may cause a decline in plasma E_2 levels and the subsequent reduction in therapeutic effects. Despite this, few people in the current study (n = 2) were taking concurrent medications that induce CYP3A4, namely carbamazepine. On the contrary, there were more participants taking medications that typically inhibit CYP3A4 (estrogen group n = 8, placebo group n = 5), specifically antidepressants such as sertraline, fluoxetine and paroxetine, which would be expected to increase plasma E_2 levels. Findings may

have also been related to estrogen's positive biofeedback regulation with LH, where moderately high estrogen levels trigger a surge in LH (usually at the time of ovulation) which then initiates the conversion of androgens to progesterone (Laycock and Wise 1996). However this is unlikely as there was no significant increase in progesterone levels after treatment. It is more likely that low plasma E_2 levels for the estrogen group are in-part related to the prolactin-inducing properties of many antipsychotic drugs (in particular risperidone) and the successive negative feedback effect that elevated prolactin has on estrogen levels (for review see Mortimer 2007). Therefore it is possible that the use of certain atypical antipsychotics by women in the current study may have altered metabolic factors and in-turn affected the absorption of E_2 into the bloodstream and the subsequent distribution throughout the body. Thus, it is possible that the use of certain atypical antipsychotics by women in the current study may have reduced the efficacy of E_2 treatment and therefore reduced the likelihood of seeing a significant effect on cognitive performance.

Old generation typical antipsychotics are widely recognized as having a negative impact on cognitive function, especially when compared to the newer atypical antipsychotics. However, findings of the current study may have been confounded by the use of different 'atypical' antipsychotics, as research now suggests certain atypicals selectively affect particular cognitive domains, possibly via potentiation of dopaminergic and cholinergic function (for review see Meltzer and McGurk 1999). Specifically, clozapine has been shown to repeatedly improve attention and verbal fluency but not working memory or verbal and spatial memory, risperidone has consistently enhanced attention, working memory and executive function, while olanzapine is postulated to selectively enhance verbal learning, verbal fluency and executive function (Meltzer and McGurk 1999). However, as specified earlier participants in the current study were still performing below normal levels, implying that any positive effects atypical antipsychotics could have had on cognition were not large enough to fully restore functioning to premorbid levels.

One reason for the current findings may be due to the sample comprising women of child-bearing age (range 17 – 49yrs). It may be that a substantial loss of endogenous estrogen is required before E_2 treatment can have a significant effect, as seen with the

effects of HT in healthy post-menopausal women. However, it should also be considered that E_2 treatment may only be useful for women of child-bearing age with a diagnosis of hypoestrogenemia, which is prevalent in approximately 58% of women with schizophrenia of this age group (Bergemann et al 2005a). It would be of interest for future research to also determine whether ET, EPT as well as treatment with selective estrogen receptor modulators (SERMs), would improve cognitive deficits in post-menopausal women with schizophrenia. It may be the disorder itself that restricts estrogen from having an effect at the cellular level, where the abnormalities in the dopaminergic, serotonergic and cholinergic systems have already occurred. This would be in line with the rationale applied to Alzheimer's Disease, where estrogen has been found to have a protective effect against the development of dementia but is essentially ineffective in the treatment of cognitive deficits, after the cellular deterioration has already taken place (Fillit 2002).

6.5 Conclusion

The current findings suggest that short-term E_2 treatment does not alleviate cognitive deficits in women of child-bearing age with schizophrenia. It may be that a longer duration of treatment or a higher dose is needed before observable cognitive enhancement becomes apparent. Alternatively, it may simply be that adjunctive E_2 is beneficial for the treatment of psychotic symptoms, but is less effective for the alleviation of cognitive deficits. However this has yet to be determined and further research into the effects of estrogen in young and middle-aged women is required before definitive conclusions can be drawn. It is also necessary to investigate whether adjunctive estrogen treatment will improve cognitive function in post-menopausal women with schizophrenia. Research into this possibility is currently underway. Further research into the underlying neurochemical mechanisms of E_2 in the human brain is required to help determine the nature of estrogen's variable effects on cognitive function and it's significant role in the pathophysiology of schizophrenia and related disorders.

SECTION FOUR:
GENERAL DISCUSSION & CONCLUSIONS

Chapter 7

Summary, Implications of Results & Future Directions

This thesis reports on the cognitive effects of short-term E_2 treatment in healthy young women and women of childbearing age with schizophrenia. The mechanisms underlying E_2's actions in the brain are largely unknown. The role of the cholinergic system in mediating the cognitive effects of E_2 was explored in the current thesis, given the prominent role of this neurotransmitter system in fundamental cognitive processes, namely attention, declarative memory and learning. These cognitive processes are commonly impaired in schizophrenia and such cognitive impairments have been suggested to arise from abnormalities in the integrity and regulation of the basal forebrain cholinergic system. Cognitive deficits have now been widely accepted as an independent symptom dimension of schizophrenia, which have debilitating effects on functional outcome. Thus, researchers are now looking to discover effective adjunctive treatments to improve cognitive functioning for individuals afflicted by cognitive deficits. Given the long line of evidence supporting estrogen's neuroprotective effects in the brain and in schizophrenia, it has been suggested that E_2 treatment may improve cognitive functioning for women with schizophrenia.

7.1 Summary of Key Findings

The aim of the first experimental study was twofold; (1) to investigate the cognitive effects of one month of 100µg/day transdermal E_2 treatment in a sample of healthy young women, and (2) to test whether short-term E_2 treatment can attenuate or protect against the cognitive deficits incurred after administration of the cholinergic muscarinic receptor antagonist scopolamine. The results showed that one month of E_2 treatment had positive effects on select areas of cognitive function but no effect on the majority of tasks. Specifically, a significant improvement was observed in spatial working memory performance on the 1-back task. In addition there was a trend towards enhanced long-term verbal recall but no effect of E_2 treatment was seen on the other measures of declarative verbal learning and memory (total words List A (trails 1-5) of the RAVLT). Furthermore one month E_2 treatment had no effect on the other cognitive domains assessed including verbal fluency, attention, cognitive flexibility and information processing and psychomotor speed.

In addition this study found that one month of E_2 treatment had no protective or attenuating effect against scopolamine on any of the cognitive domains assessed other than for the measure of delayed verbal recall (and this was only for participants in the follicular phase at the post-treatment assessment). This is generally incongruent with the only other research group to test this hypothesis in humans (Dumas et al 2006; Dumas et al 2008). Dumas and colleagues (2006; 2008) found that three months of oral E_2 treatment in postmenopausal women significantly attenuated the scopolamine-induced deficits on two measures of attention (Critical Flicker Fusion task and continuous performance task) and a measure of verbal episodic memory (Buschke Selective Reminding Task). They also found that E_2 attenuated attentional deficits caused by the nicotinic receptor antagonist mecamylamine (Dumas et al 2006). The current finding also contrasts the long line of animal research which has consistently found E_2 to protect against the cognitive impairments induced by scopolamine.

Experiment two aimed to investigate the cognitive effects of one month E_2 treatment as an adjunct to antipsychotics in women of childbearing age with schizophrenia. The results showed that E_2 treatment had no beneficial effects on any of the cognitive domains examined including declarative verbal memory and learning, verbal fluency, visual memory, working memory, cognitive flexibility, attention and information processing and psychomotor speed. Despite this, there was a significant difference found between the two groups on the domain of visual memory, where the E_2 group showed no change while the placebo group improved in performance on the immediate memory trial of the WMS III Visual Reproduction task. Lastly, there were no significant correlations found between baseline endogenous E_2 and progesterone levels with any of the cognitive measures.

7.2 General Discussion and Implications

7.2.1 Estradiol Treatment Effects on Cognition in Healthy Women & Women with Schizophrenia?

Experiment One is the first study to discover that administration of E_2 treatment in healthy cycling women of childbearing age can significantly enhance cognitive performance. To date, research has focused on the neurocognitive effects of ET in post-menopausal women and women with dementia. Surprisingly, few researchers have investigated the cognitive effects of ET/EPT (positive or negative) in young healthy women. During the mid 70s, two studies showed women taking oral contraceptives were slower on reaction time tasks and during mental arithmetic when compared to women not taking the pill (Creutzfeldt et al 1976; Wuttke et al 1975). However, a later study found no effect of oral contraceptives on a number of cognitive measures (Silber et al 1987). A large percentage of young women are currently on a hormone regimen. Specifically, approximately 29-45% of the Australian female population aged between 18 and 35 years of age use the contraceptive pill (Yusuf and Siedlecky 2007), while as many as 11.6 million women in the United States were taking the oral contraceptive pill in the year 2002 (Mosher et al 2004). Given that oral contraceptive pills contain the most potent forms of exogenous estrogens (namely ethinyl E_2 [EE]) which can raise plasma E_2 concentrations up to 1000 pg/ml with a 50µg dose of EE (de Lignieres and Silberstein 2000), it would be of interest to examine whether such a dramatic short-term alteration in normal ovarian hormonal levels would have any effect on cognitive functioning and general well-being in young women.

To speculate, the reason that cognitive effects of E_2 treatment in young healthy women has not been investigated previously may be due to the fact that this cohort does not require any 'treatment' as such, given that they are essentially healthy (i.e. with a normal menstrual cycle) and cognitively functioning at a high level. Historically, HT has been viewed as a treatment for those with a deficiency in endogenous E_2 levels, whether that be due to a genetic disorder (such as Turner's syndrome) or from the natural progression to the menopause. When estrogen was

suggested as a possible treatment for the cognitive impairment seen in dementia, including the Alzheimer's type (Fillit et al 1986), there was again no need to compare the effects to a younger sample given that the onset of dementia is around the age of 65-70 (Ropacki and Jeste 2005), well after transition to the post-menopause. From another perspective, behavioral evolutionary research, namely studies on menstrual cycle phase/endogenous hormone levels and cognitive performance, has been grossly inconsistent and would further suggest ET in healthy young women would not be of any real consequence to cognitive functioning. The reasons for the investigation of ET in women of childbearing age in the current thesis was due to: (1) the lack of knowledge regarding ET-effects in this age cohort, as it was simply not known whether additional E_2 given to healthy cycling women would have any effect on cognition, and (2) the clinically positive effects of ET in patients with schizophrenia and the proposal that adjunctive ET may improve cognitive deficits inherent to this disorder (Akhondzadeh et al 2003; Kulkarni et al 1996; Kulkarni et al 2001). In light of the findings from previous research in healthy women of this age cohort, it was surprising that in Experiment One significant, but selective positive effects of E_2 treatment were found on cognitive function, the reasons and significance of which will now be explored. Given this is the first study of it's kind, comparison with previous research is difficult, thus readers should bear in mind that interpretation of the findings are preliminary.

One thing that is clear from the behavioral literature which has investigated the neurocognitive effects of ET is that it is largely inconsistent. A myriad of researchers have reviewed this literature with some concluding that there is not sufficient evidence to suggest estrogen has any positive influence on cognitive function (Haskell et al 1997; Hogervorst et al 2002; Lethaby et al 2008; Rice and Morse 2003; Yaffe et al 1998b), while others delineated a pattern for beneficial effects on verbal memory (Hogervorst et al 2000; LeBlanc et al 2001; Sherwin 2002; Zec and Trivedi 2002) as well as abstract reasoning and information processing (Hogervorst et al 2000; LeBlanc et al 2001). Neither of the experiments in this thesis found significant positive effects on any of these three cognitive domains. Despite this, we did find working memory to improve, a domain that has infrequently been

found to be significantly affected by ET in the past (Duff and Hampson 2000; Keenan et al 2001; Miller et al 2002).

Working memory has been suggested to be the foundation of most other higher-order executive functions such as planning, abstract thinking and problem solving (Smith and Jonides 1998), and is incidentally one of the most commonly impaired cognitive processes in schizophrenia (Lee and Park 2005). Moreover, working memory deficits in schizophrenia have been suggested to be a strong contributing factor to the impaired social skills and low employment capacity of individuals with this illness (Breier et al 1991; Green 2006; Green et al 2000). Visuospatial working memory specifically can be practically defined as the ability to mentally represent and manipulate visual and spatial properties of our environment over a short period of time (a few seconds), and is an essential cognitive process used in everyday functioning. Visuospatial working memory has consistently been found to be impaired in schizophrenia and the magnitude of this impairment is large (population effect size d = -1.00), according to a recent analysis of 33 clinical studies (Piskulic et al 2007). Various types of tasks have been robust in detecting visuospatial working memory deficits, including tasks utilizing the '*N*-back' paradigm (Jansma et al 2004) as used in Experiment One. In addition visuospatial working memory performance has been related to negative symptoms of schizophrenia such as flat affect, poverty of speech, apathy and reduced spontaneous movement (Pantelis et al 2004; Pantelis et al 2001), suggesting treatment of working memory dysfunction may inadvertently improve negative symptoms of schizophrenia and visa versa. Therefore, E_2's ability to enhance spatial working memory performance in Experiment One may have implications for the treatment of working memory deficits in women with schizophrenia. Despite this, as described in Experiment Two, there was no significant E_2 treatment-effect on the working memory domain (Digit Span backwards WMS III) in women with schizophrenia. However, it may be that the Digit Span backwards task used in the latter study does not require the same level of cognitive control and degree of on-line manipulation as the n-back paradigm. Previous researchers have challenged the assertion that the Digit Span task is a true measure of working memory, with many concluding the task is primarily a general measure of attention with the backwards trial engaging only the basic aspects of

working memory (Lezak et al 2004). Alternatively, it may be that E_2 treatment selectively enhances visuospatial working memory in young pre-menopausal women and has no effect on numerical/verbal working memory. Lastly, it should be noted that participants remained on their normal regimen of antipsychotic medication throughout Experiment Two. However, although certain atypicals are known to have modest positive effects on cognition, participants were still performing well below normal levels at baseline, allowing for potential enhancement following E_2 treatment.

Disappointingly, one month E_2 treatment in women of childbearing age with schizophrenia had no beneficial effects on any cognitive domain assessed, suggesting short-term adjunctive E_2 treatment is not effective in treating cognitive deficits for women of childbearing age with schizophrenia. The findings of experiment one are partially supported by the findings of the clinical study in that the majority of tasks were unaffected. Interestingly, when the treatment group was not factored into the analysis there was a general improvement in a number of the cognitive domains assessed including declarative verbal memory and learning (CVLT), verbal fluency (COWAT), visual memory (Visual Reproduction) and information processing and psychomotor function (TMT B). These findings are possibly related to practice effects. The finding that both groups improved on these measures presents as a confounding factor, in that E_2 treatment would have needed to exert a more robust effect on these tasks for significant cognitive enhancement to have been observed over and above that associated with practice effects.

Overall, while working memory was significantly enhanced by one month E_2 treatment, with a trend towards significant improvement in long-term verbal memory (Experiment One), the majority of cognitive domains were unaffected (both Experiment One and Two). Indeed, the vast estrogen literature is rife with opposing findings, with nearly as many researchers finding no or negative effects as those who found positive effects of estrogen on the exact same cognitive domains, and often the exact same cognitive measures (see Chapter 2 for further information). Typical confounding factors, such as the 'healthy user bias' and the duration of time between menopause and initiation of HT, have previously been proposed as possible causes for inconsistent and contradictory results. However, these types of factors are

relevant only to post-menopausal women and therefore cannot explain findings in our studies comprising women of childbearing age.

A few animal-based theories have been put forth to explain why certain cognitive processes may be enhanced while others are unaffected or impaired with ET. It has been suggested that estrogen's opposing effects on different cognitive processes may be related to the type of cognitive strategy adopted to complete the given task (Daniel and Lee 2004; Davis et al 2005; Korol and Kolo 2002). Korol and Kolo (2002) were the first to suggest that estrogen's effects on cognition may be task-specific, as they found OVX rats treated with E_2 learnt a 'place' plus-maze task, which required the use of allocentric hippocampal-dependent strategies (ie. strategies related to the environment), faster than controls but were slower in learning a 'response' plus-maze task, which required the use of egocentric striatal-dependent strategies (ie. strategies which are related to ones self). These findings lead the authors to imply that ET may actually increase the tendency for adopting strategies specific to the hippocampus, regardless of whether or not the hippocampal-dependent strategy is more appropriate for the task being performed (Korol 2004). Other animal studies also support the theory that ET protects performance on tasks requiring hippocampal-dependent memory processes, but not those which are less reliant on the hippocampus and involve single representations of information, such as spatial reference memory (Daniel 2006; Daniel and Lee 2004; Davis et al 2005; Zurkovsky et al 2007). However, Korol and Kolo's (2002) theory is somewhat flawed given that numerous researchers have found reference memory to improve significantly after ET in animals (El-Bakri et al 2004; Frick et al 2002; Heikkinen et al 2004; Iivonen et al 2006), while studies in humans have found evidence for ET-enhanced frontal cortical activation (Eberling et al 2004; Joffe et al 2006; Maki and Resnick 2000; Resnick et al 1998; Shaywitz et al 1999; Smith et al 2006). In addition, research has found better performance on tasks reliant on this brain region, including working memory as found in Experiment One of the current thesis (Duff and Hampson 2000; Keenan et al 2001; Miller et al 2002), as well as abstract reasoning and response inhibition (Fedor-Freybergh 1977; Jacobs et al 1998; Joffe et al 2006; Keenan et al 2001; Krug et al 2006; Rice et al 2000; Schmidt et al 1996). Furthermore, human studies investigating ET-effects on cognition, including those in

the current thesis, have failed to find significant improvements on hippocampal-dependent tasks requiring processes such as learning/memory encoding (eg. CVLT, Paired Associate Learning), spatial memory (eg. Visual Reproduction) and declarative memory (Clock Drawing task) (Almeida et al 2006; Barrett-Connor and Kritz-Silverstein 1993; Binder et al 2001; Duka et al 2000; Kampen and Sherwin 1994; LeBlanc et al 2007; Maki et al 2007; Paganini-Hill and Henderson 1996a; Pefanco et al 2007; Shaywitz et al 2003; Wolf et al 1999; Yaffe et al 2006).

While the existence of an 'optimal' (ie. inverted U function) level of E_2 for modulating specific cognitive processes has been proposed (Hampson 1990; Nyborg 1983), findings of Experiment One suggests otherwise, as high dose E_2 to healthy cycling women did not impair any aspect of cognition and only enhanced one measure of spatial working memory. Given that the n-back task used in the current study is reliant on prefrontal cortical functioning (Ellis et al 2006; Nystrom et al 2000), it may be that relatively high levels of estrogens in the prefrontal cortex facilitate working memory. This is in line with the work of Sinopoli and colleagues (2006) who found high dose (5µg) E_2 benzoate in rodents resulted in enhanced performance on a spatial win-shift (working memory) task. However, Sinopoli and colleagues subsequently found that lower dose (0.3µg) E_2 benzoate resulted in impaired working memory performance. Moreover, they also found high dose E_2 (0.9µg/0.5µL) infused directly into the prefrontal cortex facilitated working memory while the same dose infused into the hippocampus resulted in impaired performance, suggesting a possible dose-by-region-dependent effect of E_2 treatment on working memory. Also in support of this theory is the finding that low dose E_2 (0.1µg/0.5µL) infused into the hippocampus was more effective in facilitating working memory than either the high dose or placebo (Sinopoli et al 2006), which is in line with previous animal studies that used low dose E_2 treatment and found enhanced spatial working memory performance (Bowman et al 2002; Daniel et al 1997; Fader et al 1999; Holmes et al 2002; Luine et al 1998; Rapp et al 2003). Behavioral neuroimaging studies in healthy pre and postmenopausal women also suggest estrogen may selectively influence memory and learning tasks mediated by the hippocampus as well as prefrontal-cortex-dependent tasks requiring executive functioning (Berman et al 1997; Joffe et al 2006; Maki 2005; Maki and Resnick

2000; Resnick et al 1998; Shaywitz et al 1999; Smith et al 2006; Stevens et al 2005). Given these findings, further research into the possible dose- and site-specific effects of E_2 treatment on cognitive function is warranted.

Paradoxically, while higher-than-normal estrogen levels may account for the effects on working memory, the lack of significant effects on the other cognitive domains in both experiments may also be dependent on estrogen levels as well as the age of the sample population. In comparison to post-menopausal women, where a degree of age-related cognitive decline in fluid intelligence, such as cognitive speed and memory, has invariably already commenced (usually beginning around the age of 49), young pre-menopausal women are in their peak and cognitively functioning at an optimal level (Christensen 2001; Rabbitt et al 2003). Therefore, it could be speculated that E_2 treatment in young healthy women may not exert a positive effect on neurocognitive functioning due to the absence of any cognitive deterioration, thus there is no need to restore performance back to baseline levels (i.e. ceiling effects). Similarly, healthy young and pre-menopausal women who have not gone through menopause have normal levels of estrogen and thus do not require restoration of circulating ovarian hormones as do post-menopausal women. However, as mentioned previously in Chapter 5, this theory does not fully explain why working memory was significantly enhanced in Experiment One. In addition, although women with schizophrenia were of child-bearing age, they presented with abnormally low estrogen levels in comparison to the normal population (Hoff et al 1998), which is in line with previous research suggesting that women with schizophrenia who suffer from hypoestrogenism should, like post-menopausal women, received ET to restore this loss in endogenous estrogens (Bergemann et al 2005). However, this argument is made with the assumption that ET can only be beneficial when endogenous estrogens have been depleted and does not account for the possibility that cognition may improve beyond normal baseline functioning with ET. Indeed, an animal study found sham-operated female mice given 40 days ET demonstrated enhanced learning on a RAM task when compared to control sham-operated mice, and sham-operated mice given 7 days ET (Heikkinen et al 2002). This suggests that estrogen can have a positive effect even when there has not been a decline in/or disruption to endogenous estrogen levels, which may be dependent on

duration of treatment. It is also possible that only certain cognitive processes, such as working memory, can be enhanced when estrogens are above the normal physiological range.

Molecular and brain imaging research suggests E_2 may have an effect on mental processes without causing changes in cognitive performance. Specifically, an fMRI study found the high estrogen phase of the menstrual cycle to be associated with increased activity in the inferior frontal gyrus, medial frontal gyrus and post-central gyrus during a word-stem-completion task, as well as the inferior temporal-occipital cortex and superior parietal lobule during a mental rotation task (Dietrich et al 2001). Interestingly, while there were significant changes in cortical activation, no significant differences were found in performance on cognitive tasks. It is suggested that decreases in rCBF and cortical activation (when performance remains stable) represents more efficient processing and less mental effort to perform the given cognitive task (Just et al 1996). If this is the case then Dietrich and colleagues' (2001) finding supports the theory of an optimal level of circulating estrogens where higher E_2 levels correspond with poorer performance and an increase in the mental effort required.

A PET study in healthy young women found that suppression of ovarian hormones (with leuprolide acetate; a gonadotropin-releasing hormone (GRH) agonist) resulted in reduced perfusion in the DLPFC, which was restored to normal after E_2 treatment (Berman et al 1997). Again, although there were significant alterations in cortical activation there was no observable change in cognitive performance after E_2 treatment on the Wisconsin Card Sorting Task, a reliable measure of executive functioning. This suggests that neuroimaging techniques may be more sensitive for detecting estrogen's effects on brain networks associated with cognitive functioning than are behavioral measures. In support of this, brain imaging studies in post-menopausal women have also found alterations (increases and decreases) in activation of cortical and sub-cortical areas of HT-users compared to non-users, and that these differences were not associated with any change in cognitive-task performance (Shaywitz et al 1999; Stevens et al 2005). Furthermore, Shaywitz and colleagues (1999) noted that with ET, activation patterns during working memory

tasks were similar to those observed in healthy young individuals (Cabeza et al 1997; Schacter et al 1996), suggesting estrogen may enhance plasticity of memory systems in post-menopausal women, which is in line with animal research and the neuroprotective hypothesis (for reviews see Cooke and Woolley 2005; Murphy and Andrews 2000). Therefore, although the current thesis did not find significant effects of E_2 treatment on many of the cognitive domains assessed, it is possible that E_2 treatment altered functioning at the brain network level during these tasks. However, it should be noted that the underlying significance of relative change in rCBF, quantified using fMRI, is not well understood. Whether or not the blood-oxygen-level-dependent (BOLD) signal is driven by neuronal and cellular energy usage is still under debate, with recent evidence suggesting changes in the BOLD response should be viewed as a reflection of neuronal signaling (i.e. information processing) rather than energy consumption (i.e. mental effort exerted) (Attwell and Iadecola 2002; Jansma et al 2007). Furthermore, estrogen's ability to act as a vasodilator in cerebral arteries (predominantly via modulation of endothelial nitric oxide synthase, cyclo-oxygenase -1 and prostacyclin synthase) needs to be taken into consideration when viewing MRI data (for review see Duckles and Krause 2007).

One methodological limitation in the current thesis was that plasma hormone levels were not measured in Experiment One, thus disallowing the ability to test for correlations between cognitive measures and E_2 levels, or to confirm menstrual cycle phase and test whether estrogen levels were in-fact different between the two groups after treatment. However, plasma hormone levels were measured in Experiment Two. Surprisingly there was no significant increase in E_2 level for the E_2 group from baseline, nor were there any differences between the two groups at any weekly time-point throughout the one month period, even after taking into account menstrual cycle phase at baseline. As discussed in Chapter 6 this may have been related to a number of factors, such as induction of CYP3A4 (which is the primary enzyme that metabolizes E_2) due to the use of concomitant medications. The addition of 100µg/day E_2 to young and pre-menopausal women may also alter endogenous estrogen bio-feedback effects with LH and prolactin, as high estrogen levels maintained for a period of time will induce a surge in LH which subsequently reduced estrogen levels and increases progesterone levels (Laycock and Wise 1996).

Furthermore the use of antipsychotics may have a secondary effect on endogenous hormones given that typical antipsychotics, as well as risperidone, have been shown to induce hyperprolactinemia (Kinon et al 2003), which can then cause a reduction in estrogen levels and other adverse effects. In addition, a number of the atypical antipsychotics have been found to increase weight gain and alter lipid and glucose metabolism which can lead to hypertension, low high-density lipoprotein cholesterol (HDL-C) and elevated fasting levels of serum triglycerides and glucose, all of which can have adverse effects on the cardiovascular system (for review see Meyer et al 2005). Therefore it is possible that the use of certain atypical antipsychotics by women in the current study may have altered the above metabolic factors and in-turn affected the absorption of E_2 into the bloodstream and the subsequent distribution throughout the body. Alternatively, it may be speculated that the E_2 patches were not securely fixed to the skin resulting in inadequate absorption of E_2 through the epidermis. However, this in unlikely as participants were given detailed clear instructions on how to affix the patches and, when necessary, were shown a demonstration on how to apply them. Furthermore, psychopathology significantly improved for the estrogen group compared to the placebo group, suggesting E_2 treatment was capable of exerting a significant effect on behavior. Experiment Two also included secondary analyses of the relationship between circulating plasma E_2 and progesterone levels with cognitive measures. The lack of significant correlations between E_2 and progesterone levels with cognitive function in our clinical sample further supports the likelihood of finding non-significant and unreliable results had analysis of hormone data in Experiment One been possible. A number of studies have shown circulating E_2 levels, whether from endogenous or exogenous sources, to be unrelated to cognitive performance (Almeida et al 2005; Herlitz et al 2007; Janowsky et al 2000; Portin et al 1999; Yaffe et al 1998a; Yonker et al 2003). However, an equal number of studies have found significant positive correlations of E_2 with various cognitive measures (Drake et al 2000; Farrag et al 2002; Maki et al 2002; Wolf and Kirschbaum 2002). These findings add to the grossly inconsistent literature and raises further doubt regarding the direct relationship between circulating plasma E_2 levels and behavioral cognitive measures (see Chapter 2 for review). This is further supported by recent evidence suggesting circulating plasma

E_2 levels do not accurately reflect E_2 levels in the brain, given that certain brain regions produce their own estrogens locally (Simpson 2003).

Another possible explanation for the lack of an effect of E_2 treatment on cognition, and working memory in particular (for Experiment Two), may relate to the mechanisms by which E_2 exerts its neurocognitive effects. It has been speculated that estrogens may enhance cognitive function via modulation of various neurotransmitter systems, including the dopaminergic, serotonergic and cholinergic systems (for reviews see McEwen 2001; Norbury et al 2003). Given that the majority of atypical antipsychotics target multiple receptors from these neurotransmitter systems including D_2, D_3, D_4, 5-HT_{1A}, $5HT_2$, $5HT_3$, other 5HT receptors and muscarinic M_1-M_5 receptors (Meltzer and McGurk 1999), it may be that adjunctive E_2 was competing with the actions of antipsychotic medications at these receptor sites or with typical antipsychotic medications at predominantly D_2 receptor sites (Cravchik et al 1999), in key brain regions essential for cognitive functioning. However, this is quite speculative and assumes E_2 affects cognition via interaction with one or a combination of these neurotransmitter systems. Thus, Experiment One of the current thesis investigated one possible mechanism via which E_2 may act, the cholinergic system.

7.2.2 Role of the Cholinergic System in Mediating Estrogen's Cognitive Effects

The lack of significant protective effects of short-term E_2 treatment against the anticholinergic, scopolamine-induced cognitive impairments in Experiment One (part 2) may be due to a number of factors. As discussed previously, 'age' may be a critical factor influencing the effects of E_2 on cognition. The natural process of aging is accompanied by a progressive decline in fluid intelligence (in particular episodic memory, memory span, verbal fluency, comprehension and psychomotor speed) with a dramatic drop in performance occurring around the age of 40 (for reviews see Christensen 2001; Rabbitt 1993; Salthouse 2003). It has been suggested that this drop in performance may be related to the loss in cholinergic functioning associated with normal aging given that research has found dramatic decreases in the number and size of p75 and ChAT immunolabeled neurons of the cholinergic basal forebrain (Smith et al 1999), and decreases in cholinergic innervation (AChE axon terminal

density) of various cortical regions, including the frontal and cingulate cortices of aged rhesus monkeys (Conner et al 2001). A more recent study also found aged Long-Evans rats displaying impaired spatial learning had a 23% loss of cholinergic neurons (immunostained for ChAT) in the medial septum/dbB compared to young animals, and this loss was directly correlated with poor spatial learning performance (Baskerville et al 2006). Interestingly, they found that a third group of aged rats, which performed similarly to young animals, showed no decline in cholinergic innervation, implying a causal relationship between cholinergic cell loss in this region of the basal forebrain with age-related impairments in learning abilities. In addition, human studies have also found a significant positive correlation between cell loss in the nbM and advancing age (Szenborn 1993), as well as lower muscarinic receptor density in the striatum, frontal, temporal and parietal cortices, hippocampus, and cingulate cortex of post-menopausal women compared to young women (Norbury et al 2007). These findings are further supported by the cholinergic degeneration inherent to AD and related dementias, disorders characterized by severe cognitive impairment (for reviews see Francis et al 1999; Sirvio 1999). The theory that E_2 treatment may only be beneficial to the cholinergic system when cholinergic functioning is impaired is in line with the long line of evidence suggesting women who use HT after the menopause have a significantly lower risk of developing AD in older age (for review see Fillit 2002).

It may also be that E_2 treatment only has protective effects on cholinergic neurons when endogenous estrogens have been depleted, such as that occurring with the menopause. Indeed, as discussed in Chapter 3 there is ample evidence to support this theory with previous animal studies finding ovariectomy to be related to; (1) a significant drop in ChAT mRNA, which can be restored with E_2 treatment (Gibbs 1998; Gibbs et al 1994; McMillan et al 1996), (2) a decrease in muscarinic receptor binding (Vaucher et al 2002), (3) a decrease in cholinergic fiber density in the pre-frontal cortex of monkeys 2 years post-ovariectomy (Tinkler et al 2004), (4) upregulation of muscarinic receptors in the hippocampus (Cardoso et al 2004; El-Bakri et al 2002), and (5) increased AChE in numerous brain regions (Das et al 2002). Behavioral animal studies that have tested the protective effects of E_2 treatment on the cholinergic system by administering scopolamine to OVX rats have

consistently found positive effects on cognitive performance (Dohanich et al 1994; Fader et al 1998; Fader et al 1999; Gibbs 1999; Gibbs et al 1998; Savonenko and Markowska 2003; Tanabe et al 2004), further suggesting a relationship between endogenous estrogen levels and cholinergic functioning (see Chapter 3 for review). Unfortunately, no study has administered a cholinergic challenge to young gonadally-intact estrogen-treated animals, thus the OVX animals used in these studies had essentially gone through a similar processes as human surgical menopause, making these studies less relatable to healthy young women of child-bearing age. Similarly, human studies linking estrogens' effects to the cholinergic system have also only focused on women of the 40+ demographic. Despite this, findings are promising as Norbury and colleagues (2007) found post-menopausal women taking ET had higher muscarinic receptor density in the left striatum, hippocampus, lateral frontal cortex and thalamus than women who had never used ET. Smith and colleagues (2001) also found higher VAChT binding indexes in the posterior cingulate cortex of post-menopausal women taking ET as opposed to EPT-users. Furthermore, although they did not find any difference in binding indexes between users and non-users there was a significant positive correlation between number of year of HT use and VAChT binding indexes in the frontal, parietal, temporal and cingulate cortices. These findings, along with those of Dumas and colleagues (2006) as discussed earlier, support the hypothesis that a dramatic drop in endogenous estrogens coupled with the loss of cholinergic innervation associated with aging, may be a prerequisite for ET to significantly protect or enhance cognitive processes. In addition, this may be further dependent upon how soon after the menopause treatment is initiated, given that Dumas and colleagues (Dumas et al 2008) found the protective effect ET had on verbal memory after a cholinergic challenge, was only observed for younger as opposed to older postmenopausal women (for further discussion of the 'critical period hypothesis' see Sherwin 2007).

The role of the cholinergic system in fundamental cognitive processes is well established as outlined in Chapter 3, however cognitive function is modulated by a number of other neurotransmitter systems. It is possible that estrogen's cognitive effects may be mediated via the dopaminergic or serotonergic systems, especially seeing as working memory was significantly enhanced by E_2 treatment in experiment

one after one month of treatment but was not protected against the impairing effects of scopolamine.

A role for dopamine is suggested on the basis of supporting evidence from animal research which has found estrogen to; (1) upregulate dopamine receptors in striatal neurons and neuroblastoma cells (Hruska and Silbergeld 1980; Lee and Mouradian 1999), and (2) attenuate the gonadectomy-induced reduction in axonal density and tyrosine hydroxylase immunoreactivity of dopamine neurons in the prefrontal cortex of male rats (Kritzer 2000; Kritzer et al 2007). These findings, together with human studies showing better dopaminergic responsivity and increased serum dopamine levels in postmenopausal women taking estrogen (Craig et al 2004; Zarate et al 2002), and the well-established fundamental role of dopamine in working memory and executive functioning (for reviews see Chudasama and Robbins 2006; Goldman-Rakic 1996; Seamans and Yang 2004), suggest that estrogen may modulate cognitive processes via a dopaminergic mechanism. Similarly the serotonergic system, implicated in declarative memory and learning (for review see Meneses 1999; Schmitt et al 2006), has also been proposed as a modulatory mechanism for estrogen's effects on cognition. Specifically, research has shown; (1) females to have greater impairment in declarative memory than males when 'tryptophan', the central serotonin precursor, is depleted causing a reduction in 5-HT synthesis (Sambeth et al 2007), (2) estrogen to stimulate an increase in the density of 5-HT_{2A} receptor binding sites in numerous brain regions including the frontal cortex of animals and humans (Fink et al 1996; Kugaya et al 2003; Moses et al 2000; Sumner and Fink 1995), (3) ET to decrease density of serotonin reuptake transporter mRNA in the dorsal raphe nucleus of macaques (Bethea et al 2002; Pecins-Thompson et al 1998), and (4) ET to decrease 5-HT_{1A} receptor binding in the hippocampus of female rats (Osterlund et al 2000), consistent with the reports of a negative relationship between hippocampal 5-HT_{1A} receptor binding or activation with memory and learning (Carli et al 1995; Yasuno et al 2003). Despite this however, the exact role of 5-HT and its receptors in learning and memory functions yet to be fully characterized. Further, given the complex interactions between serotonin and other systems including the cholinergic, glutamatergic and GABAergic systems, interpretation of the modulatory effects of estrogen on the serotonin system in relation to cognition is speculative at this stage and requires further investigation.

7.3 Future Directions

7.3.1 Weighing up the Risks and Benefits of Estrogen Treatment

In light of the premature cessation of the Women's Health Initiative (WHI) randomized controlled trial, due to the increased incidence of invasive breast cancer (Rossouw et al 2002), it is necessary to discuss the need for weighing up the risks and benefits of estrogen treatment. To date epidemiological studies and randomized controlled trials have concluded HT increases the risk of venous thromboembolic events by 2- to 5-fold in postmenopausal women (Davison and Davis 2003; Rossouw et al 2002; Wu 2005), which parallels findings regarding oral contraceptive use in women of child-bearing age (Gomes and Deitcher 2004). It is important to note however that these large-scale extensive reviews all reported that the increased risk was specific to EPT, with the general consensus being that there is a significantly smaller risk of thrombosis associated with estrogen-only treatment, and that there is no conclusive evidence to suggest transdermal E_2 has any increased risk at all. Furthermore, a number of other risk factors that contribute to an increased risk of thrombosis, such as obesity, hypertension, diabetes, smoking and hyperlipidaemia are not always controlled for and should be taken into consideration (for review see Davison and Davis 2003). Similarly, research has consistently shown an increased risk of breast cancer (by up to 53%) in EPT-users with prolonged treatment (i.e. > 5 years) (Beral et al 1997; Colditz et al 1995; Ewertz et al 2005; Fournier et al 2005; Lund et al 2007; Rosenberg et al 2006; Ross et al 2000; Rossouw et al 2002; Schairer et al 2000; Stahlberg et al 2004), with most finding a significantly smaller risk associated with estrogen-only preparations (Colditz et al 1995; Fournier et al 2005; Ross et al 2000; Rossouw et al 2002; Schairer et al 2000; Stahlberg et al 2004). This is consistent with a recent meta-analysis of 13 studies (Shah et al 2005). A number of studies have also found a significantly higher incidence of endometrial cancer in HT-users (Jain et al 2000; Lacey et al 2005; Newcomb and Trentham-Dietz 2003; Pike et al 1997; Reed et al 2004; Weiderpass et al 1999).

The Women's Health Initiative Memory Study (WHIMS), which utilized a subgroup of women from the WHI study, surprisingly found that CEE either alone or in

combination with a progestin, significantly increased the risk of dementia and cognitive decline after approximately 5 years of treatment (Craig et al 2005). This contradicts the numerous observational studies that suggest ET protects women from developing AD (Costa et al 1999; Paganini-Hill and Henderson 1996b; Tang et al 1996; Waring et al 1999; Zandi et al 2002). The findings of the WHIMS may be related to the age at which women began treatment (range 65-79 years), which was well past the menopausal years and as previously mentioned, increasing evidence suggests ET may only be beneficial when initiated soon after the menopause (for discussion see Sherwin 2007). Despite the increased risks associated with HT use, it has been found beneficial for preventing osteoporosis. This is based on a meta-analysis of 57 randomized controlled trials and a recent review of low-dose combined EPT, which concluded that HT consistently and substantially protects against bone density loss (van de Weijer et al 2007; Wells et al 2002). Similarly, the WHI study found EPT to significantly reduce the number of fractures compared to the placebo group (Rossouw et al 2002).

Overall, long-term use of combined EPT is not recommended for disease prevention (Tattersall 2002), however the relative risks associated with short-term HT-use or transdermal E_2 treatment is undetermined at this stage and requires further investigation. Given the findings of the current thesis it would appear that E_2 treatment is not effective or appropriate for the treatment of cognitive deficits in women of child-bearing age with schizophrenia, however this is not taking into account the possible benefits of ET on psychopathology and positive symptoms which have been found to significantly improve with short-term ET (Akhondzadeh et al 2003; Kulkarni et al 1996; Kulkarni et al 2001), and may well outweigh the risks involved. Further research into the cognitive effects of varying durations of ET is needed before definitive conclusions can be drawn. The aims of the current thesis were to examine the efficacy of the most potent form of the natural estrogens (E_2) on cognition and determine whether estrogen exerts its effects via modulation of the cholinergic system. In addition, we also aimed to investigate whether adjunctive E_2 in general would be a viable treatment for the cognitive deficits seen in schizophrenia, with the implication that more suitable centrally acting safer drugs

could be tested/produced had E_2 treatment been proven effective. Indeed, alternative, safer options have already been proposed.

7.3.2 Alternative Treatment Options for Cognitive Deficits in Schizophrenia

Two proposed alternatives to traditional ET are phyto-estrogens and the recently developed SERMs (namely tamoxifen and raloxifene), compounds which have both agonistic and antagonistic effects in the brain and have been found to be neuroprotective (for review see Zhao et al 2005). Phytoestrogens are non-steroidal plant constituents containing naturally occurring sterols that bind to estrogen receptors (ERs) and structurally resemble endogenous estrogens, yet they are considered much safer than traditional ET preparations given they are considerably less potent (Ruggiero and Likis 2002). In addition, phytoestrogens have been found to have a higher binding affinity for ERβ than ERα (for review see Patisaul 2005). Given the ERβ subtype has been suggested to be the more prominent ER in mediating effects on cognition (McEwen 1999), such findings provide further support for exploration of phytoetrogens as possible cognitive-enhancers. Although biochemical evidence and behavioral animal research supports soy isoflavones (the most commonly known phyto-estrogen) as having positive effects on brain function, human studies have found mixed results (for review see Lee et al 2005). The Soy and Postmenopausal Health in Aging (SOPHIA) study found 6 months treatment with 55mg/day soy isofavones significantly improved category fluency, but not verbal memory or information processing (Kritz-Silverstein et al 2003). Another research group that also used a randomized double-blind placebo-controlled design found an equivalent dose of soy isoflavones supplement for 6 and 12 weeks improved sustained attention, non-verbal short-term memory, mental flexibility and planning of healthy post-menopausal women (Duffy et al 2003; File et al 2005). Despite this, an equal number of double-blind placebo-controlled randomized studies have shown no significant positive cognitive effects of 4-12 months treatment with soy isoflavones (Fournier et al 2007; Ho et al 2007; Kreijkamp-Kaspers et al 2004). Similarly, a randomized controlled clinical trial of aglycone isoflavones (derived from red clover) also found no significant effects of 6 months treatment on cognition (Howes et al 2004). Collectively these findings show that there is presently

insufficient evidence to support a prominent effect of phyto-estrogens on cognitive function.

SERMs are also considered a safer alternative to traditional estrogen preparations given they are nonsteroidal drugs designed to target selective ERs in specific tissue types, primarily used for the treatment and prevention of breast cancer (Swaby et al 2007). However, investigations into the cognitive effects of SERMs have yielded mixed results. Research in post-menopausal women has shown raloxifene (60mg/day) to have no significant effect on cognition after 8 weeks (Haskell and Richardson 2004), 3 months (Neele et al 2001) or 1 year of treatment (Yaffe et al 2001), although Neele and colleagues (2001) did find a significant difference in brain activation patterns during a visual encoding task in comparison to a control group. A pilot study which also used 60mg/day raloxifene for 3 months actually found a decrease in attention as measured by a letter-search task (Natale et al 2004). Similarly, a pilot study of tamoxifen in breast cancer patients found a negative effect on immediate verbal memory (Jenkins et al 2004), however this was in comparison to healthy post-menopausal women as within-group comparisons were not possible (plus treatment length varied between participants, range = 12-60 months). In line with this are the findings of Eberling and colleagues (2004) who also showed patients receiving tamoxifen performed worse on a semantic memory task compared to healthy controls. Despite these negative findings the Multiple Outcomes of Raloxifene Evaluation (MORE) randomized placebo-controlled trial found 3 years of high-dose (120mg/day) but not low-dose (60mg/day) raloxifene significantly reduced the risk of developing mild cognitive impairment in post-menopausal women with osteoporosis (Yaffe et al 2005). Similar dose-specific improvements have been observed on delayed verbal memory after 1 month of treatment, although there were no significant effect at 6 or 12 months (Nickelsen et al 1999). In summary, the current potential cognitive effects of SERMs are largely speculative and require further investigation. It should also be noted that not all SERMs are the ultimate replacement for traditional ET, as tamoxifen in particular is associated with increased risk of endometrial cancer (Neven and Vergote 2001). Thus, the development of molecules that selectively target and activate ER-mechanisms in the brain, while concurrently avoiding ER-activation in peripheral tissue, is a growing area of

research and will undoubtedly lead to the creation of novel NeuroSERMs in the near future.

With more specific relevance, the National Institute of Mental Health (NIMH) Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) Initiative has put forth promising neuropharmacological targets for drug development (for discussion see Green 2007). These targets were: α-7 nicotinic receptor agonists, D_1 receptor agonists, AMPA and NMDA glutamatergic receptor agonists, metabotropic glutamate receptor agonists, α-2 adrenergic receptor agonists, glycine reuptake inhibitors, M_1 muscarinic receptor agonists and $GABA_A$ receptor subtype selective agonists (for reviews see Arnsten 2004; Barch 2004; Coyle and Tsai 2004; Friedman 2004; Goldman-Rakic et al 2004; Lewis et al 2004; Marenco and Weinberger 2006; Martin et al 2004; Moghaddam 2004). These proposals have predominantly been based on animal models, although some cholinergic and glutamatergic agents have been trialed in schizophrenia, including donepezil, galantamine, glycine, D-serine, D-cycloserine and the AMPA-receptor positive modulator CX516 (see Chapter 6 for further information). However, over half of the clinical trials that have used these compounds have failed to find any improvement in cognition, further supporting the need for the development of novel pharmacological agents. Compounds that promote enhanced cholinergic or dopaminergic functioning, in particular α-7 nicotinic receptor agonists and D_1 receptor agonists respectively, appear to be the most popular area of research for the future treatment of cognitive deficits in schizophrenia (Green 2007).

The Federal Drug Association (FDA)-NIMH-MATRICS workshop in April 2004 produced a set of guidelines for designing clinical trials aimed at assessing novel adjunctive agents for the treatment of cognitive deficits in schizophrenia (Buchanan et al 2005). It was concluded that future research should aim to ensure novel agents be administered in conjunction with a specific antipsychotic, based on the pharmacokinetics, binding affinities and modes of action of the two drugs. It is likely that certain novel compounds may interact synergistically with particular antipsychotic medications to improve cognitive function. It was suggested that this possible interaction be explored after safety and pharmacokinetics have been

established and following proof-of-concept of the agent. Secondly, future research should endeavor to limit the trial of novel compounds to patients who are in the residual phase of the illness and should exclude patients who are performing at or near ceiling levels on baseline cognitive screening tasks (Buchanan et al 2005). However, the latter is not likely to occur often, given that research shows 98% of patients are cognitively impaired when taking into consideration pre-morbid functioning (Keefe et al 2005).

Lastly, given that a recent meta-analysis evaluating the effectiveness of Cognitive Remediation (CR) Therapy in schizophrenia found significant improvements (effect size = 0.41) in cognitive functioning (McGurk et al 2007), CR Therapy should also be explored further as an adjunct to antipsychotic pharmacotherapy and traditional Cognitive Behavioural Therapy (CBT). Worth noting however, cognitive performance is not the only predictor of functional outcome as social cognition, defined as the mental operations that involve the perception, interpretation and processing of social information (Ostrum 1984), has recently been suggested to be a better predictor of functional outcome than neurocognition (Brune 2005; Pinkham and Penn 2006; Vauth et al 2004). Therefore, other psychosocial therapies, such as Social Cognition and Interaction Training (SCIT) (Penn et al 2007), which focus on improving social cognition, should also be explored further. Due to the heterogeneity of schizophrenia it is important to acknowledge that no one treatment is going to be suitable for every patient, and at the clinical service level it will be necessary to identify the form of pharmacological treatment and/or psychosocial therapy that will best meet the needs of the individual patient.

7.4 Concluding Remarks

While there is plausible evidence to suggest that various neurotransmitters including acetylcholine, dopamine and serotonin may all be pathways via which estrogen exerts its neuroprotective effects in the brain, research is still a long way from determining what effect these interactions have on cognitive functioning. Indeed the astounding biochemical evidence of E_2's neuroprotective mechanisms, from adult neurogenesis to antioxidant effects, suggests E_2 should exert positive effects on

memory and executive functioning, which is grounds for further exploration of this field despite the grossly inconsistent behavioral literature. Future research should specifically focus on controlled randomized double-blind experiments over a 12-month treatment period, combined with neuroimaging techniques. Further biochemical and molecular research is also needed to delineate the exact nature of E_2's interaction with neurotransmitter systems and the subsequent effects on cognitive performance in both young and postmenopausal women.

The current thesis produced the novel finding that working memory can be enhanced with short-term E_2 treatment in a sample of healthy young cycling women. Despite this, the majority of cognitive domains were unaffected by E_2 treatment. The overall findings of the current thesis further suggest that short-term E_2 treatment does not protect against or attenuate the cognitive impairing effects of the muscarinic antagonist scopolamine, nor is it effective as an adjunct for treating cognitive deficits in women of child-bearing age with schizophrenia. However, given these are two of the few studies to have investigated the cognitive effects of E_2 in this age cohort, further research is required before definitive conclusions can be drawn. Based on the long line of evidence supporting E_2's neuroprotective effects in the brain and in schizophrenia, adjunctive E_2 for the treatment of cognitive deficits in post-menopausal women with schizophrenia should be explored further. The recent acknowledgement that cognitive deficits are a core symptom of schizophrenia has undoubtedly revolutionized the perceived treatment of this disorder and brought forth a shift in focus for research in this field. Consequently, the search for discovering effective treatments for alleviating cognitive deficits in schizophrenia is in its infancy and will certainly expand and diversify in coming years.

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Table of Estradiol and Estrone Concentrations Observed with Different ET Preparations in Postmenopausal Women (pg/ml)* *(adapted from Dören, 2001, p35)*

Preparations	Daily dose (mg)	Estradiol (pg/ml)	Estrone (pg/ml)
Oral			
Micronized Estradiol	1	30-50	150-300
	2	50-180	300-850
Estradiol Valerate	1	50	160
	2	60-70	185-300
CEE	0.625	30-50	150
	1.25	40-60	120-200
Parenteral			
Estradiol patches	0.05	30-65	40-45
	0.1	50-90	30-65
Gel	1.5	40-100	90
	3.0	60-140	45-155
Vaginal			
Micronized estradiol	0.5	250	130
CEE	1.25	25-40	65-80

CEE – conjugated equine estrogens, * Approximate concentrations reported using conversion factor: pmol/l divided by 3.67 = pg/ml

Appendix B. Summary Table of Cognitive Behavioral Research Involving Ovariectomized Female Animals Treated with Estrogen

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Singh et al. (1994)	Sprague-Dawley rats (12)	E_2/placebo	N/R (5mm s.c.s.c)	2 or 25 weeks	Active avoidance & Morris water maze (learning/ spatial reference memory)	E_2 enhanced learning on the active avoidance task at both time points.
O'Neal et al. (1996)	Sprague-Dawley rats (30)	Polyestradiol phosphate/placebo	0.5mg s.c injection every 3 weeks for 200 days	48 hrs (testing ever 6 weeks)	Spatial DMS water maze task (working memory)	Estrogen enhanced working memory for the 5min delay trial relative to controls.
Packard & Teather (1997)	Long-Evans rats (41)	Estradiol-cyclodextrin/ Placebo	0.1, 0.2 or 0.4 mg/kg i.p	24 hrs	Water maze (memory)	All E_2 groups had better memory than controls. The 0.2 dose was more effective than 0.1 & 0.4.
Fader et al. (1998)	Long-Evans hooded rats (16)	EB/placebo	5µg/kg s.c daily injections	1-3 weeks	T-maze (learning)	E_2 enhanced learning performance but not rate of acquisition.
Fader et al. (1999)	Long-Evans hooded rats (24)	E_2/placebo	N/R (5mm s.c.s.c)	30-60 days	RAM (spatial working & reference memory)	E_2 enhanced working memory and learning.
Gibbs et al. (1999)						
Experiment 1:	Sprague-Dawley rats (12)	E_2/placebo	N/R (3mm s.c.s.c)	10 days	T-maze, DMP task (spatial memory)	E_2 had no effect.
Experiment 2:	Sprague-Dawley rats (24)	E_2/placebo	N/R (3mm s.c.s.c)	2 & 11 months	DMP task (learning)	E_2 enhanced learning of the DMP task at 2 months but not 11 months post treatment.
Voytko (2000)	Macaca fasicularis monkeys (13)	E_2/placebo	N/R (35 mm s.c.s.c)	1 week – 5 months, & at 16 months	OD, spatial DR task (learning/ memory)	E_2 had no effect.

Appendix B continued…

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Gibbs (2000a)	Sprague-Dawley rats (111)	E_2/ E_2 + P /placebo	N/R (5 mm s.c.s.c) E_2 or 10µg E_2 + 500µg P/week injections	6-8 weeks or 7 or 10 months	T-maze, DMP task (learning)	E_2 alone or combined with P enhanced learning, with E_2 + P being most effective.
Galea et al. (2001)	Long-Evans rats (82)	EB/placebo	10µg/day	as long as necessary to reach criterion (up to 32 days)	RAM tasks (spatial working & reference memory, place & response learning)	High dose E_2 impaired reference memory, stimulus response learning & conditioned place learning.
Bowman et al. (2002)	Sprague-Dawley rats (32)	E_2/placebo	N/R (10 mm s.c.s.c)	33 days	RAM (spatial working memory)	E_2 enhanced memory, regardless of whether rats received chronic restraint stress.
Das et al. (2002)	Sprague-Dawley rats (10)	Estradiol dipropionate/ Placebo	1µg/daily s.c	8 days	Passive-avoidance test (learning)	E_2 enhanced learning in the passive avoidance test.
Frick et al. (2002)[2]	Aged C57Bl/6 mice (28)	EB/placebo	1 or 5µg/daily s.c injection	5-10 days	Spatial and cued water maze tasks (working & reference memory/learning)	5 µg but not 1 µg EB enhanced spatial learning and memory. No effect was seen on reference memory.
Fry & Rhodes (2002)						
Experiment 1:	Long-Evans rats (200)	E_2/ placebo	N/R (s.c.s.c) or 1mg or 10µg s.c injection	3 days	Inhibitory avoidance task (learning/memory)	All E_2 regimens enhanced performance. All regimens were equally effective.
Experiment 2:		E_2/ placebo	1µg implant into h.c. (or nucleus accumbens)	3 days	Inhibitory avoidance task (learning/memory)	E_2 implanted directly into the hippocampus enhanced performance.

Appendix B continued …

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Experiment 3:		E_2/Tamoxifen/ E_2+Tamoxifen/ placebo	5µg/day E_2 s.c &/or 10 mg/kg Tamoxifen s.c injections	3 days	Inhibitory avoidance task (learning/memory)	Tamoxifen did not block the performance enhancing effect of E_2.
Experiment 4:		E_2/Tamoxifen/ E_2+Tamoxifen/ placebo	1µg E_2 implant to h.c &/or 10 mg/kg Tamoxifen implant to hippocampus	3 days	Inhibitory avoidance task (learning/memory)	Intrahippocampal E_2 when administered alone, enhanced learning & memory.
Experiment 5:		E_2/cyclodextrin/ ICI 182,780/ Placebo	1µl E_2 &/or 1µl ICI 182,780 or cyclodextrin infusions to h.c	3 days	Inhibitory avoidance task (learning/memory)	Intrahippocampal E_2 when administered alone or with ICI 182,780, enhanced learning & memory.
Experiment 6:		E_2:BSA/ Placebo	N/R implant to h.c	3 days	Inhibitory avoidance task (learning/memory)	E_2:BSA implants enhanced learning & memory performance.
Heikkinen et al. (2002)	Female & male C57Bl/6J mice (121)	E_2/placebo	0.18 mg subcutaneous minipellets	7 or 40 days	T-maze, RAM (learning & working memory)	E_2 enhanced RAM acquisition in OVX, non-OVX and gonadally-intact male mice. E_2 enhanced T-maze learning in OVX female mice. 40 days E_2 was more effective than 7 days.
Korol & Kolo (2002)	Sprague-Dawley rats (42)	EB/placebo	2 x 10µg injections	48 hrs	Plus-shaped maze task (place & response learning)	E_2 treated rats learnt the place task faster than controls. The opposite was found with the response task.
Lacreuse et al. (2002)	Aged Rhesus monkeys (5)	EE & raloxifene & placebo (in alternating 28-day blocks)	450 ng/kg/day EE 1 mg/kg/day of raloxifene	9 months (daily testing 5 days/week)	DR task, DNMP task, spatial DRST (memory)	E_2 transiently enhanced spatial working memory performance. There was no effect of raloxifene on any task.

Appendix B continued …

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Markowska & Savonenko (2002)	Middle-aged Fischer-344 rats (30)	E_2/placebo	3 x 10µg injection = 1 set, 4 sets in total, plus 5mm s.c.s.c	1 day – 3 months (repeated testing)	DNMP task, water maze tasks, (working & reference memory)	Chronic E_2 treatment improved working memory only with adjunctive injections of E_2.
Vaucher et al. (2002)	Young & old C57Bl/6J mice (88)	E_2 (no placebo)	N/R (5mm s.c.s.c)	21 days	Object recognition (memory)	Both young & old E_2 treated mice showed greater recall than OVX age matched controls.
Voytko (2002)	Young Macaca Fascicularis monkeys (18)	E_2/placebo	N/R (3.5cm s.c.s.c)	1 week- 4 mths (monthly testing), & at 14 mths	Visuospatial cuing task (attention), simple RT, spatial DR task (memory)	No between group differences were found. Spatial attention improved from baseline for the E_2 group at the 4 month time-point.
Lacreuse & Herndon (2003)						
Experiment 1:	Young Rhesus monkeys (6)	EE & placebo (alternating 28-day blocks 7-day blocks)	450 ng/kg/day	8 months (tested 5 days/week)	Match-to-sample task, spatial, object & face DRST (memory)	E_2 impaired performance on the face-DRST. There were no effects on the other tasks.
Experiment 2:		EE & placebo (alternating 7-day blocks)	450 ng/kg/day	4 months (tested 4-5 days/week)	face DRST (memory)	E_2 impaired memory/processing for conspecifics' faces only.
Foster et al. (2003)	Young, middle-aged & aged Fischer-344 rats (75)	EB/placebo	N/R (high dose = 1cm s.c.s.c; low dose = < 0.5cm s.c.s.c)	21 days	Inhibitory avoidance task, Morris water maze cue & spatial discrimination tasks (learning/memory)	High dose EB impaired memory on the inhibitory avoidance task. Memory for spatial cues was better for young, middle-aged & aged rats given placebo, low-dose EB & high dose EB respectively.

Appendix B continued …

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Rapp et al. (2003)	Aged Rhesus monkeys (16)	Estradiol cypionate/ Placebo	100µg every 3 weeks	23 days approx.	DR (spatial working memory), DNMS (object recognition), OD	E_2 treated animals performed better than controls in the DR task, & DNMS task (when intermediate delays were used).
Tanabe et al. (2004)	Sprague-Dawley rats (41)	E_2/ E_2 +P/ P (no placebo)	1.5 mg E_2 &/or 200 mg P (s.c 90-day release pellets)	3 weeks	RAM (spatial working & reference memory/ learning)	E_2 & E_2 +P groups had improved acquisition compared to the P group & OVX controls.
Daniel et al. (2005)	Long-Evans hooded rats (48)	EB/placebo	10µg x 2 (injections)	3 days	Morris water maze (working memory)	Memory was enhanced for EB-treated rats compared to OVX controls.
Davis et al. (2005)	Long-Evans rats (NR)	E_2 (no placebo)	0.5mg (60-day release pellet)	7-10 days	8 arm RAM (place & response learning, working memory)	E_2 enhanced place learning & spatial reference memory but impaired response learning, relative to controls.
Iivonen et al. (2006)	C57Bl/6J mice (82)	E_2/placebo	20µg daily i.p. injections or 0.18mg (s.c. 90-day release pellets)	20 min (phasic) -5 weeks (tonic)	RAM (reference & working memory), T-maze (memory)	Tonic E_2 enhanced reference memory in comparison to controls.
Sinopoli et al. (2006)						
Experiment 1:	Long-Evans rats (24)	EB/placebo	0.3µg or 5µg daily injections	4 hrs (daily testing for 17 days)	Delayed spatial win-shift task (learning/ working memory)	Low-dose EB impaired working memory & high-dose EB enhanced learning.
Experiment 2:	Long-Evans rats (30)	Estradiol cyclodextrin /placebo	0.1 or 0.9µg (hippocampal or PFC infusions)	40 min (daily testing)	Delayed spatial win-shift task (working memory)	Low-dose & high-dose estrogen to hippocampus & PFC respectively, improved working memory relative to placebo (which impaired performance).

Appendix B continued …

Reference	Subjects (*N*)	Treatment	Dose [1]	Duration prior to testing	Cognitive task (domain assessed)	Outcome
Hao et al (2007)	Young Rhesus monkeys (16)	Estradiol cypionate/ Placebo	100µg every 3 weeks	Approx. 2 yrs (repeated testing)	DR (spatial working memory)	Estrogen had no effect.
Zurkovsky et al. (2007)	Young Sprague-Dawley rats (41)	E_2 3-sulfate/ placebo	0.5µM (hippocampal or striatal infusions)	2 days	Y-shaped RAM (place & response learning)	Infusions to the h.c. enhanced place learning relative to placebo (which impaired learning). Infusions to the striatum impaired response learning only when extra-maze cues were removed.

Note: E_2 – 17β-estradiol, N/R – not reported, DMS- delayed matching to sample, s.c.s.c – subcutaneous silastic capsule, i.p – intraperitoneally, s.c – subcutaneous, EB- Estradiol Benzoate, P- progesterone, si – systemic injection, , OVX – ovariectomized, RAM – radial arm maze, EE- ethinyl estradiol, DR – delayed response, DRST – delayed recognition span test, DMP- delayed matching-to-position, DNMP – delayed non-matching to position, DNMS –delayed non-matching to sample, E_2:BSA – 1,3,5(10)-estratrien-3,17β-diol-6-one 6-CMO:BSA, OD – object discrimination, RT – reaction time, MS – Medial Septum, SAP – 192 IgG-saporin. [1]type of placebo is not specified however all studies used a placebo/control suitable for their study design. [2]Frick et al (2002) used aged gonadally intact female mice as ovariectomy would have been dangerous given their age (26-27 months), all other experiments used ovariectomized animals.

Study: Estrogen, the Cholinergic System and Cognition

Date: _____

Name: _____

D.O.B.: _____

Address: _____

Phone: _____

Instructions: These questions are designed to help us understand any medical problems that you may have. All information given will be treated in the strictest confidence. Please tick all relevant boxes. Please ask for assistance if you are unsure about any of the questions.

Medical History: Are you allergic to anything that you know of?

Medications?	☐ Yes	☐ No
Foods?	☐ Yes	☐ No
Surgical Tapes?	☐ Yes	☐ No
Any other substances?	☐ Yes	☐ No

If yes, please give details

Do you take any medications (prescription or over-the counter)? ☐ Yes ☐ No

If you answered yes, please fill in the details in the table below.

Name of medication	Dose	Number of times taken each day	Date of commencement

Do you have any of the following medical problems?

Heart problems?	☐ Yes	☐ No
High or low blood pressure?	☐ Yes	☐ No
Respiratory problems?	☐ Yes	☐ No
Stomach or intestinal problems?	☐ Yes	☐ No
Liver problems?	☐ Yes	☐ No
Kidney or urinary problems?	☐ Yes	☐ No
Diabetes?	☐ Yes	☐ No
Anaemia or blood disorders?	☐ Yes	☐ No
Epilepsy or fitting?	☐ Yes	☐ No
Eyesight problems or colour blindness?	☐ Yes	☐ No
Cancer?	☐ Yes	☐ No
Skin disorders?	☐ Yes	☐ No
Anxiety or depression?	☐ Yes	☐ No
Any other psychological problem?	☐ Yes	☐ No

If you answered yes to any of the questions above, please give details

_____

_____

Have you ever had any operations? □ Yes □ No
If yes, please give details

_____

_____

_____

When did you last consult a doctor? And for what reason?

_____

_____

_____

Are you, or could you be pregnant? □ Yes □ No Are you breastfeeding? □ Yes □ No

Are your periods regular? □ Yes □ No Last period ended on _____ (date)

Period usually lasts for _____ days, every _____ days.

Do you take the contraceptive pill □ Yes □ No Brand name _____

Do you follow any special diet? □ Yes □ No

If yes, what type? _____

How many glasses of alcohol do you drink? _____ glasses / day / week Type_____

Do you smoke? □ Yes □ No Number of cigarettes / day _____

Do you drink coffee? □ Yes □ No Number of cups / day _____

Do you use glasses? □ Yes □ No Contact lenses? □ Yes □ No

Do you use a hearing aid? □ Yes □ No

Do you use any other type of prosthesis? □ Yes □ No

Medical History

Study: Estrogen, the Cholinergic System and Cognition

Date: _____

Name: _____

D.O.B.: _____

Study No.: _____

Sex : **Male** ☐ **Female** ☐

Background / concurrent disease: _____

Medications _____

	YES	NO	If yes, give details below
Allergic History	☐	☐	_____
Cardiovascular	☐	☐	_____
Ophthalmologic	☐	☐	_____
Respiratory	☐	☐	_____
Gastrointestinal	☐	☐	_____
Hepatobiliary	☐	☐	_____
Renal / Genitourinary	☐	☐	_____
Metabolic / Endocrine	☐	☐	_____
Neurologic	☐	☐	_____
Musculoskeletal	☐	☐	_____
Dermatological	☐	☐	_____
Hematological	☐	☐	_____
Neoplastic	☐	☐	_____
Other (specify)	☐	☐	_____

Signature _____

Physical Examination

Name of Study:

Participant name and number:

Date:

	Normal / abnormal		comments
Chest	☐	☐	
Heart	☐	☐	
Abdomen	☐	☐	
Nervous System	☐	☐	
Lymph nodes	☐	☐	
ENT and Eyes	☐	☐	
Extremities	☐	☐	
Skin	☐	☐	
Other	☐	☐	

Baseline Obs:

BP standing _____ **BP** sitting _____

Pulse _____ **T°** _____

Height _____ **Weight** _____

Comments:

_____

_____

_____

_____

Signature _____

SWINBURNE UNIVERSITY OF TECHNOLOGY

SWINBURNE UNIVERSITY OF TECHNOLOGY
INFORMATION SHEET

The Effects of Estradiol on Cognitive Function in Healthy Pre-menopausal Women and the Role of the Cholinergic System

Research Investigators: Cali Bartholomeusz: BA, BAppSci(Hons),
Dr. Pradeep J Nathan: BSc (Hons), PhD, MRACI, C.Chem,
Prof. Jayashri Kulkarni: MBBS, FRANZCP,
Anthony De Castella: BAppSci(Hons)

Aim of the Study:

The aim of this study is to investigate the effects of estrogen on cognitive processes, such as memory, learning and attention. Estrogen is one of a group of female sex hormones produced by the ovaries. There are a number of different types of estrogen but the one used in this study is called estradiol. The ways in which estrogen affects the brain and cognition are unknown. It has been suggested that estrogen enhances memory and other cognitive processes by acting on a chemical in the brain called acetylcholine. Acetylcholine acts upon two types of chemical receptors, one type being muscarinic receptors. Acetylecholine has for a long time been associated with memory, learning and attention. A lack of acetylecholine is one of the most prominent deficits seen in Alzheimer's disease. In this study we will examine the effect estrogen has on cognitive processes after 4 weeks of treatment. We will then administer a drug that temporarily blocks muscarinic receptors and test whether or not estrogen will protect against cognitive deficits usually seen after administration of this drug.

What does this study require of you?

We are seeking your participation in this study, which will be conducted at the Brain Sciences Institute, (Swinburne University of Technology).

If you participate in this study, we ask that you attend three different recording sessions at the Brain Sciences Institute, as well as a medical screening prior to testing. The first test session will last approximately 2 hours. You will be asked to complete a series of computer memory tasks as well as a set of paper and pencil memory tasks that will be administered by one of the research investigators. You will then be randomly allocated into one of two groups. One group will receive adhesive estrogen patches (which release estrogen at a rate on 100µg every 24hours) and the other group will receive adhesive placebo patches (placebo patches look identical to the estrogen patches however they do not release any substance at all).

This study will be conducted under double blind conditions. What this means is that neither you, nor the investigator, will be aware of which group you are in. Only the senior investigator will be aware of which drug is administered during each trial. This procedure is necessary to maintain the integrity of the results. Therefore, you will be required to change the patches (whether they be estrogen or placebo patches) every 4 days for 31 days. This is because the

treatment must be constant in order to achieve best results. On day 28 you return to the Brain Sciences Institute for the second test session. This session will last for approximately 4.5 hours. You will again perform the same memory tasks that you competed in the first test session. Upon completion of these tasks a research nurse will then give you an injection which will contain either 0.4 mg of a drug called scopolamine (which temporarily effects the way that the brain chemical acetylcholine works) or a placebo (which is salty water). Again neither you, nor the researcher will be aware of which drug you are receiving, only the senior researcher will know. 1.5 hours after having the injection, you will be asked to complete the cognitive tasks again, so we can examine the effects of the drug. After this test session you are to continue taking the patches as you have been for the past 4 weeks. On day 31 you return for the third and final test session. This session will be exactly the same as test session 2, however you will receive the opposite drug treatment (either scopolamine or placebo) to the one you received on the second test session.

It is important to continue replacing the skin patches every 4 days right up until the end of test session 3, as we are trying to see whether estrogen has a protective effect against scopolamine which will be seen through your performance on the cognitive and memory tasks.

This study is similar to HRT (hormone replacement therapy) studies, and although the current study is only looking at the short term application of estrogen, recent studies have shown that there are some risks associated with long term use of HRT.

There is information about these drugs further on in this information sheet, and you should also find attached some more technical information about both drugs.

DISCOMFORT AND POSSIBLE HAZARDS

You may experience some discomfort following the administration of the drugs. Information about the drugs and their side effects are detailed below. Medical staff will be on call for the duration of each test session
You may also experience boredom and/or drowsiness during the experiment. Television will be provided during breaks.

ESTRADIOL

Estradiol (a form of estrogen) is usually administered for the alleviation of menopausal symptoms such as hot flushes, mood disturbances, sleep disturbances, urogenital atrophy etc. It is also commonly used in oral contraceptives, to treat threatened abortion, ovarian disease and osteoporosis. The use of adhesive skin patches for the delivery of estrogen is the most affective form of administration and provides more natural replacement of estrogen, for it is absorbed directly into the bloodstream. The patches raise the estradiol level to similar concentrations seen during the early to midfollicular phase of ovulation and maintains this level continuously until the patch is removed.

How Estradiol Works

Estradiol targets receptors inside the cells of a number of organs, including the uterus, vagina, urethra and mammary glands as well as regions in the brain. It can also effect cardiovascular, skeletal, immune and central nervous system cells. In these cells estradiol provokes a series of intracellular reactions and activates the synthesis of DNA and proteins.

Side Effects
Adverse side effects commonly associated with estrogen are usually related to long term administration and are therefore not applicable to this study. However a few side effects that are common (occur in 1-10% of women) include mild irritation of the skin at the site of the patch, vaginal bleeding and breast tenderness. Nausea, abdominal cramps, headaches or migraines are uncommon (between 0.1-1% chance of occurrence).

SCOPOLAMINE

Scopolamine is a drug used to treat motion sickness, gastrointestinal spasms, and in dental surgery to reduce excess saliva. In tablet form it is sold over-the-counter as the drug Kwells. However, it is more reliably absorbed when injected into the muscle of the shoulder.

How Scopolamine Works
Scopolamine is a muscarinic receptor antagonist. What this means is that scopolamine blocks the muscarinic receptor, which prevents acetylcholine from acting at these same receptors. This effectively decreases cholinergic (acetylcholine) transmission.

Side Effects
Adverse effects that have been reported following treatment with Scopolamine include dry mouth, nausea, vommitting, slight decrease in heart rate, sedation, irritability, confussion, dizziness and constipation. These adverse effects are generally seen after chronic treatment (which we are not doing in this study), but may also occur when a single dose is given (which we are doing in this study). However, any side effects are likely to be mild with administration of a single acute dose.

For your information, the average dose of Scopolamine normally prescribed is between 0.3 – 0.6 mg, 3 to 4 times a day – which is a total of between 0.9 – 2.4 mg per day. In this study a single dose of 0.4 mg will be administered.

You may also experience some discomfort following the intramuscular injection.

TESTING PROCEDURES

All testing sessions will be conducted at the Brain Sciences Institute, 400 Burwood Rd, Hawthorn.

Timetable for test session 2 and 3

Test Day

8.45 am:	Arrival of Researcher and Subject.
9.00 am:	Baseline physiological measures (ie. heart rate, temperature and blood pressure). First cognitive testing session
10.30* am:	Drug administration
10.30 pm:	break for 90 minutes (entertainment- books, videos etc.)

12.00 pm: second cognitive testing session
1.30 pm: Testing completed. Subject leaves.

for the 1st test session only, subjects would finish testing at this time and be given the skin patches with instructions for use.

ADDITIONAL INFORMATION

Prior to testing

It is also important that you do not eat breakfast on the morning of the testing session. Breakfast will be provided at the Brain Sciences Institute when you arrive for testing. It is also important that you have not consumed alcoholic or caffeinated beverages in the previous 24 hours. It is important to realise that alcohol may interact with the medications you will receive. As a result it is important that you do not consume any alcohol for 24 hours prior to the testing session, and that you also refrain from consuming alcohol for the rest of each recording day.

What if I can't make it to a session?

You are asked to let the researcher know if you are experiencing difficulty attending the sessions. This will allow the researcher to make another time with you, or to discuss the difficulties you are having. This also enables the researcher to inform the doctors who would be on call for your session, and to free the recording room for other researchers. Furthermore, if at any stage you decide to withdraw from the study completely, you are free to do so.

Transport

Due to the possibility that scopolamine may cause side effects including dizziness and sedation it is advised that you do not drive to the 2nd and 3rd recording sessions. The maximum effects of these pharmaceuticals occur between one and three hours after administration, but effects may last for a few hours beyond that. For this reason it is not advisable that you drive yourself home after the recording sessions. You will need to arrange transport home after each recording, but if this is difficult taxi vouchers will be provided from the Brain Sciences Institute if required (for transport home only).

Exclusion Criteria

If you are menopausal, we ask that you not participate in this study. We also ask that individuals who are smokers or who suffer from heart disease, peptic ulcers, diabetes, epilepsy, or who have a history of neurological or psychiatric illness do not participate in this study. Also, if you are currently on medication, are using natural therapies or have experienced any of the following you must not participate in this study:

- malignancies or history of familial breast cancer or fibrocystic mastopathy
- hyperlipidemia
- cardiovascular or endocrinologic disease
- drug or alcohol abuse
- impaired liver or kidney function

You should ask for any information you want

Please do not hesitate to ask questions about the study, or any matter about it that concerns you. People you can ask include researcher Cali Bartholomeusz (ph.9214 8291). Before deciding on whether or not to participate in the study, you may wish to discuss the matter with a relative, friend or with your local doctor. You should feel free to do this.

Other

Please note: You are free to withdraw from this study at any time, without consequence.

The results from this study may appear in publications or be provided to other researchers, but your identity will be kept strictly confidential.

Should you have any questions regarding this study, please do not hesitate to ask the researchers whom you have met today. Alternatively, any further enquiries regarding the study can be directed to the Senior Investigators, Dr. Pradeep Nathan on 9214 5216 of the Brain Sciences Institute. If you have any complaints or queries that have not been satisfactorily answered, please write to:

The Chair
Human Research Ethics Committee
Swinburne University of Technology
P O Box 218
HAWTHORN. VIC. 3122
Phone: (03) 9214 5223

SWINBURNE UNIVERSITY OF TECHNOLOGY
BRAIN SCIENCES INSTITUTE

HUMAN RESEARCH ETHICS COMMITTEE

CONSENT FORM FOR INVOLVEMENT OF

PARTICIPANTS IN RESEARCH

PROJECT TITLE

The effects of estradiol on cognitive function in healthy pre-menopausal women and the role of the cholinergic system.

RESEARCH INVESTIGATORS

Cali Bartholomeusz: BA, BAppSc (Hons) and Dr. Pradeep Nathan: BSc (Hons), PhD, MRACI, C.Chem, Prof. Jayashri Kulkarni: MBBS, FRANZCP

I,……
(Name of participant)
agree to participate in a research project entitled: The Effects of Estradiol on Cognitive Function in Healthy Pre-menopausal Women and the Role of the Cholinergic System, conducted by Dr. Pradeep Nathan and Ms.Cali Bartholomeusz.

My agreement is based on the understanding that:

- I have not had any familial or personal history of epilepsy.
- I am not menopausal.
- I do not suffer from heart disease, peptic ulcers or diabetes.
- I am not currently taking any medication or using natural therapies.
- I do not have any history of psychiatric illness.
- I have been given a full explanation and a copy an the information sheet outlining the purpose of this study,
  the procedures involved, and what I will be expected to do.

- I have been given an explanation of how estradiol and scopolamine work and have been informed about the possible side effects.

- I am satisfied with the explanation provided, relating to this project in so far as it affects me.

- I have read (or had read to me, as appropriate), and understood the information provided in the Form of
  Disclosure. Any questions I have asked have been answered to my satisfaction.

- My consent to participate in this project is given freely.

- My involvement entails completing a series of memory tasks on a computer.

- My involvement includes applying and wearing adhesive patches for 31 consecutive days.

- The recording sessions will each take approximately 5 hours to complete, bar the first session which will take approximately 3 hours. There will be three recording sessions.

- I understand that I am free to withdraw from the study at any time.

- I am aware that no participant will be referred to by name in any report or publication concerning the study in
  question.

- I understand that the project will not be of direct benefit to me.

- I agree that I shall not claim to be entitled to restrict in any way, the use to which the results of this study may be put.

- I have been informed that the information I provide will be confidential.

SIGNED……DATE……..
(Participant)

SIGNED……DATE……..
(Researcher)

Randomization of Subjects to Treatment Groups and order of Scopolamine/Placebo Drug Challenge.

Subject No.	E_2/Placebo Treatment Session 1	Drug Challenge Session 2	Drug Challenge Session 3
1	A	a	b
2	B	a	b
3	A	b	a
4	B	b	a
5	A	a	b
6	B	a	b
7	A	b	a
8	B	b	a
9	A	a	b
10	B	a	b
11	A	b	a
12	B	b	a
13	A	a	b
14	B	a	b
15	A	b	a
16	B	b	a
17	A	a	b
18	B	a	b
19	A	b	a
20	B	b	a

Note: A = placebo patch, B = estrogen patch, a = scopolamine injection, b = placebo injection

Menstrual Cycle Questionnaire

Please read the following questions carefully and respond with a written answer or by circling where appropriate.

1. What was the date of the beginning of your last period? _____

2. How long is it from the beginning of one of your periods, to the start of the next period?

_____

3. How old were you when you had your first period? _____

4. Have you had any children? _____ Yes ☐ No ☐

5. Have you had any gynaecological surgery (ie. removal of ovarian cycts, hysterectomy etc)? _____ Yes ☐ No ☐

6. Are you on the oral contraceptive pill now? _____ Yes ☐ No ☐

7. Do you have regular periods now (ie. menstruation occurs at the same time each moth ± 3-4 days, and your bleed goes for the same length of time)? Yes ☐ No ☐

» If YES go to question 10, if *NO* go to question 8.

8. when did your periods become irregular? _____

9. Describe the irregularity: _____

_____

10. Do you have any of the following problems either before or during periods:

a. Abdominal cramps and pains	Yes ☐ No ☐	Before / During
b. Back pains	Yes ☐ No ☐	Before / During
c. Leg pains	Yes ☐ No ☐	Before / During
d. Headaches	Yes ☐ No ☐	Before / During
e. Feeling bloated	Yes ☐ No ☐	Before / During
f. Change in mood	Yes ☐ No ☐	Before / During
g. Breast pain	Yes ☐ No ☐	Before / During
h. Exacerbation of symptoms (eg. paranoia)	Yes ☐ No ☐	Before / During

i. Other problems (please specify): _____

_____

11. Do other women in your family have the same menstrual problems as you? Yes □ No □

12. Have you been told you have illnesses such as polycystic ovaries, pituitary tumors, thyroid problems? Please describe in detail: _____

_____

13. Are you currently taking or have you ever taken the oral contraceptive pill or any other hormonal medication? _____ Yes ☐ No ☐

If yes, how many years did you take it for? _____

14. How many times have you been pregnant? _____

Patch Information Sheet

How the patch works
When you apply the patch, it releases small amounts of estrogen in a continuous and controlled way (or it releases nothing, depending on whether you are on the placebo or estrogen treatment). This passes through your skin, directly into your blood stream and allows you to take much lower doses of estrogen than the tablet form.

How to use the patch
Use your patches exactly as instructed. DO NOT alter the dosing yourself. The patch should be worn continuously for 3 to 4 days. It is changed twice weekly on Mondays and Thursdays. Use only one patch at a time.

Where to apply the patch
Apply the patch to the skin of your lower back, upper buttock or abdomen (see Figure 1). Choose a place where the patch will be covered by clothing, as it should not be exposed to direct sunlight. To reduce the risk of skin irritation the patch should not be applied to the same place as the previous application. The skin of the application site needs to be clean, dry and free of moisturizers, powder or lotions. Do not apply the patch to breasts, skin that is already itchy or sore, areas where the skin folds (eg. waistline) or where it might be rubbed off by clothes.

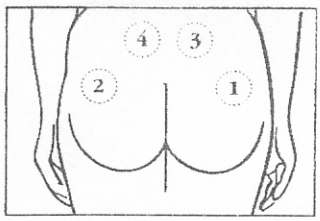


Figure 1 *Suggested application sites: 1 & 2 first week, 3 & 4 second week, 1 & 2 third week etc.*

How to apply the patch
Tear open the sachet at the notch and remove the patch. The square protective liner is separated into two parts, a small triangular tab and a larger section. Hold the triangular part between your thumb and forefinger, then remove the larger section of the liner. Still holding the patch by the triangular tab, apply the patch to the area of skin you have chosen. Remove the remaining part of the protective liner and press firmly across the whole surface of the patch for about 10 seconds. Press again with your finger along the edges to ensure good adhesion.

Other information
The patch does not need to be removed for showering, bathing or swimming. A patch should be worn at all times unless instructed otherwise. If the patch falls off replace it with a new one but continue to change patches every Monday and Thursday. After removing a patch fold it in half (sticky sides together) and dispose of it out of the reach of children.

Instructions for Spatial N-back Task

The spatial n-back task is a measure of spatial working memory.

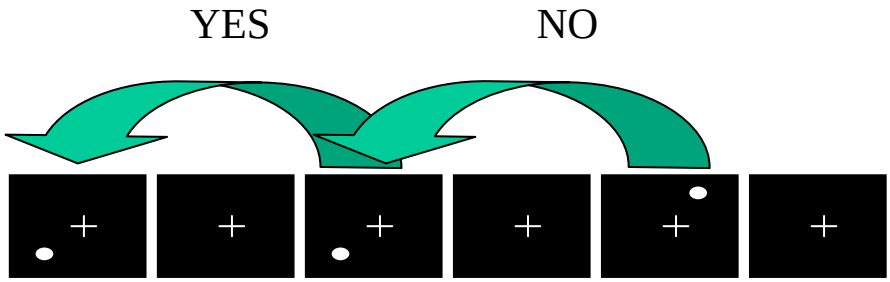
A series of dots will appear in different locations on the screen. There will be approximately 40 dots appearing one at a time. Each dot will appear for half a second and then there will be a 3 second delay before the next dot appears. During the delay a cross + will appear. This is a fixation point which you must watch until the next dot appears.

In the 1-back task the aim is to compare the location of each dot, to the dot that appeared on the screen directly before it (see below). If you think the dot is in the exact same location press the "yes" (right) button and if you think the location is different press the "no" (left) button.

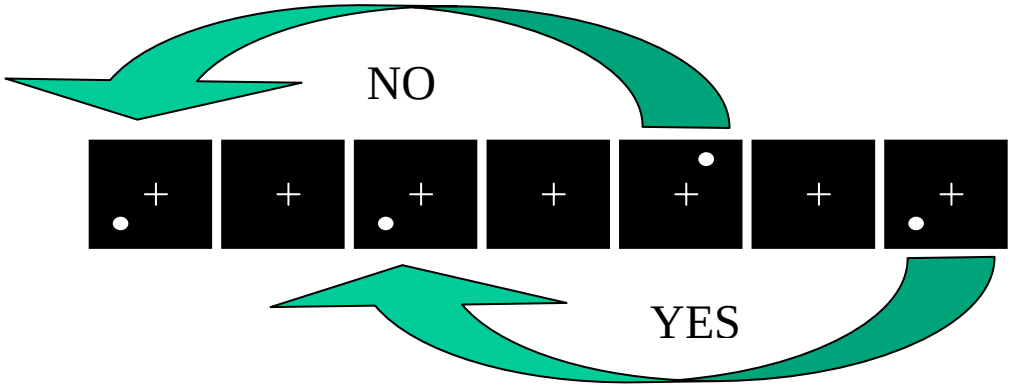
You must press only one button once for each object, even if you want to change your answer after pressing the wrong button. You should also try to make your decision as quickly as possible as your reaction time will be recorded.

The 2-back task is similar to the 1-back however the aim is to decide whether or not the dot is in the same location as the dot 2 before it (see below).

1-BACK



2-BACK



Appendix J. Means and Standard Deviations of Cognitive Raw Scores at Baseline and Post Drug Challenge (Chapter 5: Part 2)

	Estradiol group (N = 16)				Placebo group (N =14)			
	Scopolamine		Saline		Scopolamine		Saline	
Cognitive Measure	Baseline	Post	Baseline	Post	Baseline	Post	Baseline	Post
Verbal Memory & Learning								
RAVLT:								
List A total (trials 1-5)	58.81 (7.21)	48.50 (11.14)	60.13 (6.22)	58.13 (9.60)	58.79 (6.17)	47.71 (14.67)	62.13 (4.21)	59.60 (6.63)
Long-delay recall	12.81 (2.14)	7.94 (3.53)	12.81 (1.72)	11.50 (3.39)	12.36 (2.59)	7.86 (4.80)	13.00 (1.62)	12.57 (2.53)
Working Memory								
Visuospatial N-back:								
Correct: 1-back (%)	94.96 (2.56)	85.41 (8.61)	95.02 (4.00)	90.88 (6.32)	91.28 (7.76)	80.03 (9.17)	92.22 (7.41)	91.08 (6.57)
2-back (%)	88.92 (5.77)	75.96 (8.91)	86.28 (5.48)	86.98 (6.15)	82.53 (10.04)	73.00 (8.92)	85.98 (8.94)	83.30 (9.04)
RT: 1-back (ms)	547.53 (196.28)	603.51 (144.38)	548.60 (157.43)	554.61 (173.36)	571.91 (156.84)	685.12 (181.58)	539.15 (141.54)	548.92 (145.86)
2-back (ms)	604.14 (177.22)	698.23 (158.13)	631.71 (150.97)	623.89 (187.01)	620.28 (204.19)	729.73 (214.08)	592.52 (176.04)	590.33 (197.24)
Attention								
Digit Vigilance:								
Correct detection (%)	95.85 (6.11)	86.22 (14.38)	95.70 (6.14)	96.89 (3.92)	95.73 (4.58)	81.71 (11.70)	97.09 (3.99)	96.75 (5.02)
RT (ms)	403.12 (39.15)	444.46 (47.52)	412.33 (44.91)	411.54 (46.57)	424.33 (33.10)	474.68 (41.97)	409.62 (32.34)	424.89 (30.03)
Cognitive Flexibility								
STROOP:								
Interference score	9.73 (8.31)	7.57 (9.54)	8.79 (7.52)	6.43 (5.75)	7.87 (8.54)	6.64 (7.07)	8.88 (6.82)	8.99 (8.01)
Information Processing/ Psychomotor Speed								
RT: SRT (ms)	264.98 (39.44)	417.68 (287.18)	257.99 (36.13)	275.37 (47.58)	301.70 (92.52)	462.01 (290.66)	277.46 (70.77)	286.08 (61.41)
CRT (ms)	399.51 (60.36)	523.90 (214.08)	391.15 (46.95)	397.53 (50.53)	432.39 (65.31)	567.19 (269.94)	428.43 (87.76)	425.72 (91.43)
CFF (Hz)	38.07 (4.29)	35.43 (7.77)	37.36 (3.48)	37.93 (4.55)	35.67 (6.64)	35.42 (6.43)	39.17 (7.28)	38.58 (3.12)

Note: Raw scores are reported for an overall indication of actual performance, however please note analysis was conducted on "change" scores and the raw data presented here has not been screened for normality assumptions, although subjects who were excluded from the analysis due to lack of understanding of a given task have also been excluded from the calculation of the data presented here. RAVLT – Rey Auditory Verbal Learning Test, SRT – Simple Reaction Time, CRT- Choice Reaction Time, CFF- Critical Flicker Fusion.

Appendix K. Table of Means and Standard Deviations of Mood Raw Scores at Baseline and Post Drug Challenge (Chapter 5: Part 2)

	Estrogen group (n = 16)				Placebo group (n =14)			
	Scopolamine		Saline		Scopolamine		Saline	
Measure	Pre	Post	Pre	Post	Pre	Post	Pre	Post
VAMS								
Alertness	349.75 (163.15)	617.69 (192.36)	291.75 (171.62)	304.50 (192.45)	363.43 (154.35)	603.43 (144.99)	325.93 (136.95)	320.29 (120.12)
Contentedness	157.00 (63.55)	220.63 (90.96)	157.88 (88.88)	136.81 (73.64)	161.50 (84.20)	212.86 (93.04)	154.86 (73.06)	141.07 (68.37)
Calmness	69.56 (32.54)	56.25 (28.87)	66.75 (32.33)	54.31 (27.49)	64.29 (29.07)	58.14 (30.31)	58.29 (25.43)	51.00 (26.02)

Note: Raw scores are reported for an overall indication of actual performance, however please note analysis was conducted on "change" scores and the raw data presented here has not been screened for normality assumptions. Lower scores represent a greater/stronger subjective feeling of the given mood factor. VAMS – Visual Analogue Mood Scale.

Appendix L. Table of Means and Standard Deviations of Adverse Symptoms Checklist Ratings at Baseline and Post Drug Challenge (Chapter 5: Part 2)

	Estrogen group (*n* = 16)				Placebo group (*n* =14)			
	Scopolamine		Saline		Scopolamine		Saline	
Measure	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Symptoms:								
Headache	1.27 (.59)	1.33 (.49)	1.20 (.56)	1.27 (.59)	1.08 (.28)	1.08 (.28)	1.15 (.38)	1.08 (.28)
Cold	1.18 (.41)	1.82 (.87)	1.27 (.65)	1.27 (.47)	1.50 (.71)	2.20 (1.14)	1.10 (.32)	1.50 (.71)
Hot	1.31 (.48)	1.08 (.28)	1.08 (.28)	1.00 (.00)	1.23 (.44)	1.31 (.63)	1.08 (.28)	1.08 (.28)
Dizzy*	1.23 (.44)	2.77 (1.17)	1.23 (.44)	1.38 (.51)	1.00 (.00)	2.92 (1.04)	1.15 (.38)	1.23 (.44)
Increased sweating	1.23 (.60)	1.15 (.38)	1.08 (.28)	1.08 (.28)	1.08 (.28)	1.00 (.00)	1.00 (.00)	1.00 (.00)
Blurred vision*	1.14 (.36)	2.21 (1.05)	1.14 (.36)	1.14 (.36)	1.00 (.00)	2.00 (.78)	1.07 (.27)	1.00 (.00)
Nauseous*	1.00 (.00)	1.42 (.67)	1.00 (.00)	1.00 (.00)	1.00 (.00)	1.64 (1.03)	1.00 (.00)	1.00 (.00)
Heart racing	1.14 (.36)	1.14 (.36)	1.07 (.27)	1.07 (.27)	1.00 (.00)	1.00 (.00)	1.00 (.00)	1.00 (.00)
Dry Mouth*	1.38 (.96)	3.46 (.97)	1.15 (.38)	1.15 (.38)	1.38 (.65)	3.23 (.93)	1.08 (.28)	1.31 (.63)
Stomach pains	1.07 (.26)	1.27 (.80)	1.00 (.00)	1.00 (.00)	1.00 (.00)	1.18 (.60)	1.00 (.00)	1.00 (.00)

Note: * significant at p<.01.

Appendix M. Table of Raw Hormone Data for Clinical Sample at Baseline and at Day 28 (Chapter 6: Experiment Two)

Subject	Group[1]	FSH_0	LH_0	E_2_0	Prolac_0	Proges_0	Test_0	FSH_28	LH_28	E_2_28	Prolac_28	Proges_28	Test_28
1	2	5.8	13.3	209	399	16.1	1.1	5.7	11.5	248	300	14.6	1.7
2	1	2.8	3.2	278	279	16.8	1.3	6.7	3.4	92	216	0.8	1.4
3	1	27.2	49.3	448	658	6.5	1.0	22.7	16.3	155	623	1.3	1.4
4	1	1.3	1.3	360	1453	16.1	1.5	4.6	2.7	172	1810	1.0	1.6
5	1	2.6	14.1	69	2598	3.9	1.3	0.5	0.5	444	4495	0.8	1.9
6	2	3.8	3.3	285	1019	0.5	1.3	5.8	4.4	90	1020	0.5	1.3
7	1	7.4	3.6	174	367	2.6	2.1	28.3	25.8	228	658	0.7	1.7
8	1	5.8	4.8	96	206	1.0	1.1	5.5	10.5	267	143	0.8	2.4
9	2	2.8	2.5	345	144	18.3	1.2	4.2	2.3	156	285	4.8	1.4
10	1	7.4	6.0	96	860	1.4	1.7	3.6	13.0	526	567	20.7	1.8
11	2	6.1	5.1	182	291	18.3	1.4	9.1	20.3	117	370	4.9	1.9
12	2	55.8	29.3	20	176	1.4	0.9	30.8	36.9	181	377	0.7	0.9
13	1	6.5	23.5	477	239	3.6	2.1	5.9	5.0	328	271	0.7	2.4
14	1	9.5	19.8	878	195	3.4	1.7	4.9	2.4	218	137	1.0	1.4
15	1	5.9	4.2	543	793	2.5	2.7	11.8	7.9	399	435	2.3	4.8
16	2	6.1	5.4	114	277	1.6	4.0	7.4	7.6	-9	68	3.8	5.0
17	2	24.5	32.0	221	223	1.4	0.7	17.0	20.9	263	268	1.9	0.7
18	2	14.2	32.4	701	1138	5.0	1.9	12.1	11.5	212	194	12.2	1.7
19	1	24.7	19.3	103	376	1.6	0.7	15.9	27.3	235	607	1.3	1.7
20	2	5.6	11.4	644	2489	1.4	2.4	8.0	29.9	1156	960	1.4	6.2
21	2	1.7	1.8	117	4785	2.6	4.1	-9	-9	117	-9	2.6	4.1
22	1	1.6	1.8	579	177	18	1.7	6.2	6.9	424	265	0.6	1.4
23	2	7.3	10.8	214	294	2.2	3.6	7.4	11.1	261	355	0.9	1.7
24	1	4.7	17.1	1587	683	3.0	1.7	7.5	9.4	249	721	1.3	4.9
25	1	3.1	2.2	311	348	17.2	4.2	6.6	7.2	227	1178	1.0	3.7

Appendix M continued……

Subject	Group	FSH_0	LH_0	E_2_0	Prolac_0	Proges_0	Test_0	FSH_28	LH_28	E_2_28	Prolac_28	Proges_28	Test_28
26	1	4.8	9.8	481	2024	1.6	1.7	7.2	9.1	408	179	2.4	3.8
27	2	7.6	10.5	190	511	1.0	4.2	3.3	3.3	207	291	5.5	3.3
28	1	9.3	13.8	192	146	1.5	1.7	4.0	10.1	635	244	1.9	1.7
29	2	5.0	6.8	231	2482	4.3	9.0	5.4	6.2	159	2168	3.6	9.3
30	2	8.9	11.6	172	220	1.6	2.1	8.7	5.2	209	170	-9	1.7
31	2	2.6	4.7	298	4240	10.9	6.2	6.3	10.0	108	4240	0.6	7.2
32	2	6.1	14.0	282	198	15.2	2.3	3.4	7.3	251	265	14.2	1.7
33	1	7.3	7.8	184	249	1.5	2.0	3.6	7.5	571	230	2.5	3.4
34	1	-9	-9	-9	-9	-9	-9	-9	-9	-9	-9	-9	-9
35	2	2.9	3.5	524	296	32.6	3.5	4.0	7.3	460	234	49.8	2.4
36	2	5.6	4.0	201	107	0.9	2.7	6.4	9.3	208	360	2.3	2.1
37	1	6.2	12.4	583	1478	1.3	1.4	1.7	1.7	452	1731	1.8	0.7
38	1	4.0	2.0	212	945	35.8	2.6	2.9	3.9	295	1080	21.3	1.9
39	2	5.6	4.7	218	871	2.4	2.6	7.9	4.9	390	789	2.4	1.7
40	2	4.2	6.3	344	1547	23.6	0.9	8.9	36.1	745	1729	3.7	1.5
41	1	4.4	13.4	489	195	23.4	2.5	5.1	16.4	777	396	3.2	2.9
42	1	8.4	2.9	103	1913	3.3	3.1	0.7	0.2	157	3122	3.5	4.3
43	1	5.3	11.4	151	3309	3.5	2.5	3.5	4.1	103	3268	2.5	2.4
44	1	15.4	17.8	287	156	12.3	0.6	2.4	2.2	236	178	2.3	1.3
45	2	4.3	6.3	452	324	1.2	1.1	-9	-9	971	-9	-9	-9
46	2	2.8	0.7	105	1185	1.7	0.7	3.0	0.8	103	1608	1.2	0.7
47	1	41.4	25.3	315	1287	1.7	1.0	86.4	39.3	43	1034	1.2	1.0
48	1	5.7	7.2	82	691	2.8	1.8	0.1	0.1	102	1977	2.1	2.2
49	2	-9	-9	50	-9	-9	-9	-9	-9	150	-9	-9	-9
50	1	-9	-9	37	-9	-9	-9	-9	-9	121	-9	-9	-9

[1]Group 1= estrogen, 2 = placebo