Effect of Estrogen Therapy and Sex on Brain Structures in Aging: Importance of Lifelong Endogenous and Exogenous Estrogen Exposure

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<td>AG</td>
<td>amygdala</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>CEE</td>
<td>conjugated equine estrogen</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<tr>
<td>EPT</td>
<td>combination of estrogen and progestogen therapy</td>
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<td>ET</td>
<td>estrogen therapy</td>
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<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
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<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GM</td>
<td>gray matter</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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<tr>
<td>HC</td>
<td>hippocampus/hippocampal</td>
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<td>HT</td>
<td>hormone therapy</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>MPA</td>
<td>medroxyprogesterone acetate</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<tr>
<td>VBM</td>
<td>voxel-based morphometry</td>
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<td>WHI</td>
<td>Women Health Initiative</td>
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Meeting Dr. Sonia Lupien is a life changing experience most people would say. In my case, it started after an inspiring conference when I was an undergrad student. Thank you Sonia for teaching me how important it is to think inside the box to better open it and see what there is outside of it, a world of possibilities. Your passion and dedication to everything you do made this journey so enriching. Thanks also for introducing me to Dr. Jens Pruessner whom completed my formation in such a unique way. From the beginning, I knew that working with this young and ambitious researcher would be an inspiring learning experience. Thanks Jens for all these valuable teachings and countless opportunities. Thank you also for your constant listening, spontaneous laugh and boost of self-esteem! More importantly, thanks to both of you for letting me be and giving me the space I needed to find my scientific personality and future aspirations. More than once you complemented each other in such a rich way that I feel my gain was doubled to have you both on my side. Leaving your labs is not an easy thing to do but I feel that the excellent basis you gave me will carry me wherever I want to go.

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Contributions of authors

There are three manuscripts included in this thesis for which I am the first author. I have reviewed the literature, built the questionnaire named «Indices of Estrogen Exposure», collected the data, conducted data and statistical analysis, interpreted the results, and wrote the following manuscripts.

The contributions of the co-authors included supervision of the projects, design of the studies, data analysis, and advice on the style and content of the manuscripts.

1. Sonia J. Lupien and Jens C. Pruessner: Overall supervision of this Ph.D. project and contributions on writing the following manuscripts.

2. Claudia Buss: contribution to data collection, data analysis, data interpretation, and writing of the manuscript «Hippocampal Volumes are Larger in Postmenopausal Women Using Estrogen Therapy Compared to Past users, Never users and Men: A Possible Window of Opportunity Effect.»

3. Annie Duchesne: contribution to data analysis and interpretation, and writing of the manuscript «Measuring Indices of Lifelong Estrogen Exposure: Self-Report Reliability.»

4. Veronika Engert: contribution to data interpretation and writing of the manuscript «Endogenous and Exogenous Estrogen Exposure Affect Brain Regions Involved in Sexual Behavior and Memory: a Voxel Based Morphometry study.»
Abstract

Recently, new findings allow us to suggest that lifelong exposure to endogenous estrogens could be a reliable predictor of the effect of exogenous estrogens on cognition and the brain. Thus explaining why women do not arrive at menopause with an equivalent susceptibility to the effects of estrogen therapy (ET).

In the current thesis, studies have been designed to investigate the effect of estrogens on the brain from a new perspective by introducing new measures. Firstly, we developed a questionnaire about indices of lifelong estrogen exposure and assessed the reliability of these self-reported indices over a four year interval. Secondly, we recruited postmenopausal women and men to investigate the effects of estrogens on the brain using magnetic resonance imaging. Volumetric analyses of the hippocampus and amygdala and global automated analysis using voxel-based morphometry (VBM) were performed to assess whether current ET use as well as estrogen exposure throughout life can predict cerebral structural changes.

The results demonstrate that most lifelong estrogen exposure indices were reliable with age at menopause and age at HT initiation leading to the weaker correlations. An age effect on the recall of these indices was also observed. The neuroimaging results revealed that women using ET had larger HC volumes compared to past HT users, never users and men. Moreover, we found a negative relationship between ET duration and HC volume among ET users. We confirmed these results with the VBM analysis. These results support the notion that there is a treatment duration-dependent neuroprotective role of estrogen on HC volume in aging that point toward a window of opportunity. Analysing the whole brain using VBM also demonstrated that there are sex differences and that the estrogen effects are not specific to the HC since we found that ET users compared to never users have greater grey matter concentration in the nucleus accumbens and that this effect was modulated by age at menarche and age at menopause.

Overall, these results strongly suggest that lifelong endogenous and exogenous estrogen exposures have modulatory neuroprotective effect on the aging brain.
Résumé

Récemment, de nouvelles découvertes permettent de suggérer que l'exposition aux œstrogènes endogènes pourrait prédire les effets des œstrogènes exogènes sur la cognition et le cerveau, expliquant pourquoi les femmes n'arrivent pas à la ménopause avec la même susceptibilité aux effets de l'hormonothérapie à base d'œstrogènes (ET).

Dans la présente thèse, des études ont été conçues afin d'investiguer les effets des œstrogènes sur le cerveau sous un nouvel angle en introduisant de nouvelles mesures. Premièrement, un questionnaire a été développé portant sur les indices de l'exposition aux œstrogènes au cours de la vie et la fiabilité de ces indices rapportés par les participantes sur une période quatre ans a été évaluée. Dans un deuxième temps, nous avons recruté des femmes ménopausées et des hommes afin d'investiguer les effets des œstrogènes sur le cerveau en utilisant l'imagerie par résonance magnétique. Des analyses de volume de l'hippocampe et l'amygdale ainsi qu'une analyse globale utilisant de la morphométrie basée sur les voxels (VBM) ont été effectuées afin d'évaluer si l'utilisation d'ET et l'exposition aux œstrogènes au cours de la vie peuvent prédire des changements structurels cérébraux.

Les résultats démontrent que la majorité des indices d'exposition à aux œstrogènes au cours de la vie sont fiables. L'âge de la ménopause et l'âge du commencement de l'ET ont démontré des corrélations plus faibles et un effet d'âge sur le rappel de ces indices a été observé. Les résultats de neuroimagerie révèlent que les femmes qui utilisent de l'ET ont un plus gros volume hippocampique que celles qui en ont utilisé dans le passé, que celles qui n'en ont jamais utilisé et que les hommes. De plus, nous avons trouvé une relation négative entre le volume hippocampique et le durée de l'ET chez les femmes qui utilisent de l'ET. Ces résultats ont été confirmés avec l'analyse VBM. Ceci supporte la notion que les œstrogènes ont un effet neuroprotecteur sur l'hippocampe au cours du vieillissement qui est dépendant de la durée du traitement; ce qui pointe vers la notion de fenêtre d'opportunité. Les analyses du cerveau entier avec VBM démontrent aussi des différences en lien avec le sexe et que les effets des œstrogènes ne sont pas spécifiques à l'hippocampe puisque les femmes qui utilisent de l'ET comparées aux femmes qui n'en ont jamais utilisé ont une plus grande densité de matière grise dans
le noyau accumbens et que cet effet est modulé par l’âge des premières menstruations et l’âge de la ménopause.

En somme, ces résultats suggèrent fortement que l’exposition aux œstrogènes endogènes et exogènes au cours de la vie a des effets neuroprotecteurs modulatoires sur le cerveau vieillissant.
Chapter 1:
Introduction
**Introduction**

In May 2002, a major longitudinal study on the effects of hormone therapy (HT) was interrupted prematurely because the authors found that the health risks were higher than the benefits in the group of women taking HT (Rossouw, Anderson et al. 2002). This event once again raised the debate about the potential protective effect of estrogens. An especially interesting aspect of the results of the study was the observed individual differences; the question arises why some of the women had negative effects of HT while others did not.

Recently, a variable not previously considered was added to this debate: concentration and duration of life-long endogenous and/or exogenous estrogen exposure in women (Smith, McCleary et al. 1999; Ancelin and Ritchie 2005; Sherwin 2007). Estrogen exposure varies across a woman’s lifespan as a function of different factors such as age at first menstruation, pregnancies, breast-feeding, age at menopause, use of contraceptive pill and HT.

While scientists studying breast cancer and cardiovascular disease have identified a number of reproduction-related events as part of the etiology of these conditions, it is only recently that neuroscientist started to explore the potential of these events in modulating the effects of estrogens on the brain and cognition. During the past years, a lot of attention has been devoted to understand the impact of HT on cognitive function in postmenopausal women leading to mixed results. An early study attempted to link lifelong estrogens exposure to cognitive function in postmenopausal women and demonstrated that some reproductive events as well as an index pooling lifelong endogenous and exogenous estrogen exposure markers was significantly related to cognitive performance (Smith, McCleary et al. 1999). Measures of endogenous estrogen exposure prior menopause using reproductive period (age menopause-age menarche) and/or markers of greater endogenous exposure such as nulliparity and late menopause were linked to better cognitive functioning later in life (McLay, Maki et al. 2003; Dunkin, Rasgon et al. 2005; Rasgon, Magnusson et al. 2005). Regarding exogenous exposure, variables such as timing of HT initiation have been investigated, demonstrating that early initiation could be beneficial whereas initiation after a long period following menopause could lead to detrimental effects on certain cognitive functions (MacLennan, Henderson et
al. 2006). Furthermore, duration of HT is now taken into account but is leading to mixed results (Schmidt, Fazekas et al. 1996; Erickson, Colcombe et al. 2005; MacLennan, Henderson et al. 2006; Erickson, Colcombe et al. 2007). Apart from these studies, theoretical papers have been published arguing in favor of further assessment of a woman reproductive history in order to reconcile the HT findings and better characterize the effect of estrogen on the brain and cognition (Ancelin and Ritchie 2005; Maki 2006; Maki 2006; Resnick, Maki et al. 2006; Genazzani, Pluchino et al. 2007; Sherwin 2007).

The main goal of the current thesis can be divided in two parts. First, to build a questionnaire (i.e.: “Indices of Estrogen Exposure”) assessing the variables known to influence the concentration and duration of estrogen exposure throughout a woman’s life and test if these variables, when self-reported, can be reliable using test-retest procedure. Secondly, to study the effects of concentration and duration of lifelong endogenous and/or exogenous estrogen exposure on human cerebral structures using manual segmentation of magnetic resonance images (MRI) and voxel-based morphometry in a group of postmenopausal women and men.

**Factors contributing to lifelong estrogen exposure**

Estrogens are well known for their developmental effects (e.g.; sexual differentiation of the brain) during gestation but they also have a lifelong effect on the reproductive system, the cardiovascular system, bone synthesis, and the brain, through many mechanisms of action. In order to elucidate the effects of endogenous and/or exogenous estrogen exposure on the brain, factors influencing the exposure to estrogens throughout life need to be identified.

**Endogenous estrogen**

Estrogens are part of the steroid family. The steroid biosynthesis pathway begins with cholesterol as a precursor, which is then converted to other hormones such as pregnenolone, progesterone, androgens (androstenedione and testosterone), and finally estrogens (estradiol being the more potent, estrone, and estriol). The synthesis and metabolism of estrogens in women is occurring mainly in the ovaries. In addition, to a lower extent, androgens secreted by the adrenal glands are peripherally converted to estrogens in the brain, skin and adipose cells. Thus,
female's endogenous estrogens are the sum of direct ovarian secretion plus peripheral conversion. In men, the conversion of testosterone and androstenedione is the major source of circulating estrogens.

The biosynthesis in the gonads (ovaries and testes) is stimulated by a cascade of hormonal events initiated in the brain beginning at puberty and terminated at menopause in women whereas in men it is a lifelong process. Briefly, the cascade of events begins in the hypothalamus which releases gonadotropin releasing hormones (GnRH) in a pulsatile fashion and stimulates the anterior pituitary to secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which in turn stimulate the secretion of gonadal hormones (estrogen and progesterone in women/testosterone in men). In women, estrogens have a positive and negative feedback effect on the secretion of LH and FSH depending when in the menstrual cycle it occurs thus forming a closed loop system (Speroff, Glass et al. 1999; Knochenhauer and Azziz 2001; Speroff and Fritz 2005). In men, it is known that the hormonal changes are more subtle, testosterone and estrogen levels decline slowly and linearly with aging (Lamberts, van den Beld et al. 1997; Vermeulen, Kaufman et al. 2002) whereas other hormonal milestones in a woman's life can have a profound influence on the endogenous estrogen levels such as menarche, pregnancy, breast-feeding and menopause.

Menarche

Menarche is one of the later events of women puberty and is defined as the occurrence of the first menses when sufficient estrogen secretion is available. During puberty, the hypothalamic-pituitary-gonadal axis is thus reactivated; it used to be fully active during foetal life but suppressed during childhood (Speroff, Glass et al. 1999; Speroff and Fritz 2005). Following the reinstallation of a potent negative feedback, estrogen levels remain very low restraining gonadotropin secretion until 6 to 8 years of age. By age 10-11, the secretion of the gonadotropins will be resumed as a result of the reduction of the inhibition by an intrinsic suppressor of GnRH and a decrease in sensitivity to estrogen negative feedback increasing circulating estrogen levels. This event is the beginning of gonadarche, meaning the gonadal estrogen increases that will eventually lead to the first menses (menarche)(Speroff and Fritz 2005). Menarche marks the onset of cyclic changes of gonadal hormone levels. As
age of puberty onset, age at menarche is variable and influenced by genetic factors
(Meyer, Eaves et al. 1991; Eaves, Silberg et al. 2004), socioeconomic conditions
(Arim, Shapka et al. 2007), ethnicity, nutrition, weight, exercise. More recently size
at birth (Adair 2001; Tam, de Zeger et al. 2006) and exposition to endocrine
disrupters (Parent, Rasier et al. 2005; Den Hond and Schoeters 2006; Massart,
Parrino et al. 2006) were added as possible modulators. The average age of
menarche is 12.8 years, but is considered normal anywhere between ages 8 and 18
(Lee 1980; Herman-Giddens, Slora et al. 1997).

**Menstrual cycle**

During the subsequent months following menarche, the menstrual cycles are
usually irregular but will reach the usual reproductive pattern after 5-7 years of
increasing regularity. The menstrual cycle lasts on average 28 days and consists of 3
regular phases: follicular (including menses), ovulation, and luteal phases (see Figure
1). This well-established monthly pattern is characterized by different levels of
gonadal hormones among other things. The follicular phase corresponds to the first
14 days of the cycle and a sequence of events occurs that ensures ultimately that the
follicle is ready for ovulation. The first days correspond to the menstruation where
both estrogens and progesterone are low. During the early follicular phase estrogen
levels remain low, thus exerting a negative feedback on LH release. Subsequently,
they increase steadily as a consequence of follicular production. At the midfollicular
phase, there is a transition from suppression to stimulation of LH secretion following
a sustained rise in estrogens. Estrogen levels peak at late follicular hours prior
ovulation. Ovulation is characterized by a LH surge leading to the physical release of
the mature ovum from its follicular matrices and levels of progesterone rise, to most
probably terminate the LH surge. Estrogen levels on the other end plunge as LH
reaches its peak. The last phase can last from 11 to 17 days and allows the corpus
luteum to degenerate. Progesterone levels reach a peak approximately 8 days after
the LH surge and suppress the new follicular growth. The decline of the corpus
luteum involves most probably estrogens. A rise in estrogen levels is observed
accordingly during the early luteal phase prompting a fall in progesterone
concentration at midluteal. Once the corpus luteum is fully degenerated, it results in a
nadir in the circulating levels of both estrogens and progesterone allowing the
initiation of the next cycle. (Speroff, Glass et al. 1999; Knochenhauer and Azziz 2001; Speroff and Fritz 2005). ; see figure 1).

Figure 1: Schematization of different events occurring during the 28 days of the menstrual cycle at each phase (follicular, ovulation, and luteal phase). Ovarian histology, body temperature, hormonal fluctuations (FSH, estradiol, LH & progesterone), and endometrial histology are depicted.

Source :http://commons.wikimedia.org/wiki/Image:MenstrualCycle.png
Pregnancy and breast-feeding

Another endogenous factor influencing estrogen levels in a unique steadily increasing manner is pregnancy. During this period of time, there is up to a 100 fold increase of maternal estrogen excretion relative to non-pregnant concentrations. Estrogen production is dependent on fetal and placental enzymatic cooperation and is crucial in the fetal sex determination among other things (De Hertogh, Thomas et al. 1975; Speroff, Glass et al. 1999; Speroff and Fritz 2005). The estrogen concentration of this hyperestrogenic state increases with gestational age culminating prior to delivery and followed by a rapid decline and return to normal levels following parturition (De Hertogh, Thomas et al. 1975; Whitworth 1988; Speroff, Glass et al. 1999; Speroff and Fritz 2005; Snegovskikh, Park et al. 2006). This quick decline in estrogens and progesterone allow lactogenesis to occur, probably by releasing its antagonizing effect on prolactin (Whitworth 1988). Following delivery, most women go through a period of infertility associated with anovulatory amenorrhea, where estrogen levels are near post-menopausal levels (McNeilly 2001) resuming in non breastfeeding mothers 2 to 3 months postpartum (Whitworth 1988). In nursing mothers however the length of this suppressed ovarian period is believed to depend on both the central actions (hypothalamic GnRH level) of the suckling stimulus and its frequency (Whitworth 1988).

Menopause

Along with age at menarche, age at menopause is a key moment in women’s life as well as for her lifelong estrogen exposure. This period can be divided in 3 phases: the perimenopausal transition, the menopause itself and the postmenopausal years. Menstrual irregularities mark the beginning of the perimenopause on average at 46 years of age and can last from 2 to 8 years with an average duration of 5 years (Treloar 1981; den Tonkelaar, te Velde et al. 1998). During this period, estrogen levels vary unpredictably with overall levels close to normal range although they appear slightly elevated due to the increased FSH levels. This irregularity is observed up to a year prior menopause. Eventually, the gonads begin to secrete fewer estrogens, ovulation and menstruation become sparse and finally cease spontaneously on average somewhere between 50 and 52 years of age. The point in time when there is permanent cessation of menstruation is the menopause. Most definitions require
that women do not experience menstruation for at least twelve consecutive months, with no other obvious cause, to be termed menopausal. Normal menopause is known to occur between 45 and 55 years of age whereas premature menopause would occur before 40 and late menopause after 55. This unique event in a woman’s life is thought to be primarily determined by genetic factors (Snieder, MacGregor et al. 1998) with the ovary and the brain as key pacemakers (Wise, Krajnak et al. 1996; Lamberts, van den Beld et al. 1997; Genazzani, Bernardi et al. 2005). Other factors are thought to influence the onset of natural menopause such as smoking, age at menarche, parity, ethnicity, weight, diet, alcohol consumption, socio-economic status, education, use of contraceptive pill but no consensus exist on their implications (Do, Treloar et al. 1998; Kato, Toniolo et al. 1998; Speroff, Glass et al. 1999; Knochenhauer and Azziz 2001; Lawlor, Ebrahim et al. 2003; Nagel, Altenburg et al. 2005; Speroff and Fritz 2005; Dvornyk, Long et al. 2006; Parazzini 2007).

Women may also encounter menopause through surgery by the removal of their ovaries for different clinical reasons such as endometriosis and fibroids. The postmenopausal years are characterized by very low level of estrogens arising from peripheral conversion of androgens (androstenedione and testosterone) because there is no more ovarian production of estrogens (Vermeulen 1976; Lamberts, van den Beld et al. 1997; Speroff, Glass et al. 1999; Speroff and Fritz 2005).

This estrogen deprived state has an impact in women’s life, both psychologically and physiologically, and this on several systems including the bone, cardiovascular, immune, and central nervous systems. There is great heterogeneity in the manifestation of this particular hormonal state ranging from nearly no apparent changes to severe multiple symptoms. A wide range of symptoms have been reported and are difficult to attribute to estrogen deficiency solely. However, vasomotor symptoms (hot flushes) are viewed as the hallmark of the menopausal years. Physiological changes such as urogenital organ atrophy are frequent as well and may lead to sexual dysfunctions and even incontinence in some women. Other symptoms such as mood disturbances (mood swings, anxiety, depression, and irritability), cognitive-memory complaints, sleep problems (insomnia), sexual problems (decreased arousal, dyspareunia, decreased frequency, decreased libido), and somatic symptoms (aches and stiff joints) have been reported but further investigations are
needed to uncover the specific etiology of these symptoms (Woods and Mitchell 2005).

See figure 2 for a schematic representation of women’s lifelong endogenous concentration and duration of estrogen exposure.

Figure 2: Schematic representation of women’s lifelong endogenous concentration and duration of estrogen exposure.

Exogenous estrogen

Another factor influencing estrogen concentration and exposure is the exogenous administration of this hormone. Two types exist that are relevant in this context: hormonal birth control agent and hormone therapy (HT) often prescribed following menopause. Typically, the hormonal birth control agent is a combination of estrogens and progestogens or one or the other (see Frye 2006 for a review). HT is either a combination of both estrogens and progestogens (EPT) or estrogens only (ET).
Hormonal contraception

Hormonal contraception is prescribed mainly as a birth control method but can also be used for other clinical reasons such as acne, dysmenorrhea, and control of menstrual bleeding among other things. Since its first appearance on the market in the late fifties, beginning of the sixties, premenopausal women have used it for several years throughout their reproductive years. In the evolution of the oral contraceptives to their current forms, an effort has been made to decrease side effects and improve effectiveness and compliance. The first change was a decrease in the dose of estrogen and progestin leading to the low dose formulations used today. More recently, new delivery systems were introduced such as transdermal patches, vaginal rings, subcutaneous implants, and intramuscular injections (Frye 2006).

Since its introduction, the pill is the most commonly used birth control method with alternative delivery forms gaining in popularity. Depending on their hormonal components, hormonal contraceptives prevent pregnancy by inhibiting ovulation and/or modification of the endometrium preventing fertilization, and/or implantation of an egg (Speroff, Glass et al. 1999; Speroff and Fritz 2005). Oral contraceptives consist more commonly of a combination of estrogen and progestin delivering constant levels of both hormones (monophasic) or increasing the concentration of estrogen and/or progestin throughout the pill-taking schedule (biphasic or triphasic). The estrogen component of the combined or estrogen only contraceptives consist of ethinyl estradiol or mestranol; both are active forms of estradiol when given orally or transdermally. The first generation of oral contraceptives contained 50 μg or more of ethinyl estradiol compared to less than 50 μg of either ethinyl estradiol or mestranol used in the low dose today. The progestational agents in the combination or progestin only hormonal contraceptives are either norethindrone or active parent of it such as norgestimate, norelgestromin, norgestrel, levonorgestrel, desogestrel, etonogestrel, and ethynodiol diacetate or more recently, derivatives of progesterone such as medroxyprogesterone acetate (MPA). The doses of progestins vary greatly depending on the component, the combination or not with estrogens and the route of administration but rarely exceed 1mg daily (Speroff, Glass et al. 1999; Speroff and Fritz 2005; Frye 2006).
Hormone therapy

It has been over a century that ovarian steroids are used as hormone therapy (HT) to alleviate symptoms associated with menopause (Speroff, Glass et al. 1999; Speroff and Fritz 2005; Stefanick 2005). The constant conversation regarding health risk-benefit of HT between researchers and clinicians was shocked few years ago, in 2002, following the publication and media coverage of the Women Health Initiative (WHI) Study results arguing that HT causes more harm than benefit on one’s health. In 1999, Speroff starts his chapter on postmenopausal hormone therapy in his book (Speroff, Glass et al. 1999) with: « *We suggest treatment with estrogen for all women disturbed by the symptoms of hormone deprivation and advocate hormonal prophylaxis against osteoporosis and cardiovascular disease.* [...] *The recommendation that hormone therapy be given for the shortest period of time appears to be shortsighted in view of the impressive evidence that sustained therapy has a profound impact on osteoporosis and cardiovascular disease, and that there are more beneficially than potentially harmful effects.* » illustrating the general scientific opinion about HT at that time.

However, soon after the publication of the WHI study results suddenly HT began to be considered as a dangerous drug giving rise to a lot of confusion and controversies which led the future of HT at a crossroad. As a consequence an important critical re-evaluation took place in general and of the WHI results specifically (Machens and Schmidt-Gollwitzer 2003; Strickler 2003; Speroff and Fritz 2005; Speroff 2007). New guidelines were issued (for reviews see: Burger 2003; Speroff and Fritz 2005; Speroff, Kenemans et al. 2005; Stefanick 2005; Lobo, Belisle et al. 2006) and innovative research avenue are now undertaken.

New variables were introduced to better characterize and understand the effect of HT on health and the brain. Variables such as HT duration, time of HT initiation, type of estrogen-progestin used and its particular pharmacokinetic, potential differential effect of cyclic vs constant regimens are now taken into account (Ancelin and Ritchie 2005; Sherwin 2007). As in the hormonal contraception field, attempts have been made to improve effectiveness while reducing side effects and increasing compliance leading to lower dosage and various route of administration such as oral, transdermal (skin patches), topical (creams-gels), vaginal ring, and even as an intranasal spray.
HT can either be unopposed estrogen (ET) or a combination of estrogens and progestins (EPT). Dosage may vary sequentially, with estrogens taken daily and progestins taken daily only two weeks per month, or remained continuous with estrogens only or both types of hormones taken daily. The daily dose of estrogen vary from 0.3 up to 2.5 mg and can either be conjugated equine estrogen (CEE) (several sodium salts from the sulfate esters of estradiol, estrone and estriol mainly), micronized estradiol, estropipate, or esterified estrogens (Speroff, Glass et al. 1999; Speroff and Fritz 2005; Stefanick 2005). The progestin regimens combined to some HT vary greatly in dose (0.35 mg up to 5mg daily) depending on the type; the commonly used one being medroxyprogesterone acetate (MPA), micronized progesterone and norethindrone. (Speroff, Glass et al. 1999; Speroff and Fritz 2005; Stefanick 2005).

**Estrogens and the brain**

After establishing that circulating estrogen levels varies lifelong depending on the sex and different endogenous and exogenous factors, we need to establish how estrogens act on the brain. Most of studies on estrogen action in the central nervous system have focused on sexual differentiation and reproduction targeting regions such as the hypothalamus. However, the last two decades have been rich in evidence that the actions of estrogen are not restricted to the areas involved in reproductive function. Recent evidence from animal and clinical studies demonstrate that estrogens have a plethora of effects on the brain including actions on cerebral regions involved in learning and memory, emotions, and even motor coordination. There are at least two ways in which estrogens can act on the brain and have an impact on its integrity. First, estrogens might confer resilience against neuronal damage by promoting the activity and maintaining the synaptic connections functional since it has been observed that certain functions decline as estrogen levels decrease and can be restored if estrogen is added back. The second way, involve blocking neurotoxic agents or inhibiting their generation (McEwen 2001). This can be achieved via interaction with the classical intracellular estrogen receptors or through novel estrogen action which are receptor independent.
Genomic and non-genomic signalling mechanisms

Steroids such as estrogens are small lipophilic molecules that readily cross the blood-brain-barrier in their unbound state and act on the brain through several mechanisms and interactions throughout life. The classical genomic pathway of the steroid hormone involves intracellular receptors, in this case estrogen receptors alpha and beta (ERs). This traditional pathway implies that estrogens bind to its intracellular receptor and then to its estrogen response elements (EREs) located in the nuclear DNA. The bound ERE may activate in turn the expression of any genes that reside in it or a cascade of gene interactions. These ERs are expressed in large quantity with great overlap between the two subtypes, in both males and females of different species (rats, rhesus monkey and humans), in structures involved in hormonal regulation and sexual behaviour such as the hypothalamus, the preoptic area and the anterior pituitary. Interestingly, their concentration is also remarkable in areas involved in learning and memory such as the amygdala, the hippocampus, the basal forebrain and the frontal cortex (Pau, Pau et al. 1998; McEwen and Alves 1999; Osterlund, Gustafsson et al. 2000; Osterlund, Keller et al. 2000; Shughrue and Merchenthaler 2000; Shughrue and Merchenthaler 2000). (for review see (McEwen and Alves 1999; McEwen 2002))

Furthermore, it has been observed that estrogens have time-dependent effects, given that prolonged absence of hormones and chronic activity of the nuclear ER have both been shown to lead to decline in functionality (Foster 2005). In the same line of thought, differential consequences of constant versus fluctuating estrogen levels on the brain have been observed with continuous administration of HT following menopause possibly contributing to trigger pathological aging or through interaction with the immune system exacerbating normal dysfunctions associated with aging (Marriott and Wenk 2004).

Recently, some data suggested that other ERs exist such as the membrane-associated estrogen receptors allowing us to explain rapid non-genomic effects of estrogens on the hippocampus for example (McEwen, Akama et al. 2001). Even though they are currently not well understood, functional evidence suggest their importance in neural target cells of estrogen (Toran-Allerand 2004). Estrogens binding to this receptor would lead to enhanced cytoplasmic enzyme activity, activation of second messengers systems probably the same used by growth factors
and neurotransmitters. Other estrogen actions happening rapidly, also independent of intracellular ERs, occur by the activation of distinct intracellular signalling cascade. Overall, such actions of estrogens consist of rapid excitability of hippocampal neurons by estrogens, activation of cyclic adenosine monophosphate (cAMP), PI3K, and mitogen activated protein kinase (MAPK) pathways, modulation of G-protein coupling, effects on calcium current, and protection of neurons from oxidative stress (McEwen and Alves 1999; Brinton 2001; Behl 2002; McEwen 2002). This plethora of genomic and non-genomic effects explains briefly the regulation of the effects of estrogens on the brain which can either be organizational or activational and even neuroprotective.

Organizational and activational effects

Sex steroids namely estrogens, progestogens and androgens have profound effects during critical developmental years of the central nervous system. Estrogens are crucial for the sexual differentiation of the brain to occur. At the beginning of its life, the brain is bipotential, neither male nor female, until the gonads starts secreting a certain hormonal profile during a critical period. During this fetal-neonatal critical period, estrogens and aromatized androgens modulate neuronal development and formation of neural networks specific to female and male influencing the phenotype of the brain irreversibly. This is reflected by differences in the organisation and functionality of male and female brains later on. In a nutshell, this hormonally mediated sexual differentiation is hypothesized to occur through regulation of apoptosis of certain cells in brain regions destined to be sexually different and by efficient pruning of synaptic connections thereby tuned in response to specific stimuli determining the appropriate response profile later in life (McEwen and Alves 1999; McCarthy and Konkle 2005). Therefore, estrogen organizational effects are described as permanent actions during a critical early developmental period (perinatal and/or puberty) determining the brain responses to certain stimuli later in life. Estrogens are known as well for their activational effects which refer to a transient behavioural and/or physiological response triggered by the activation of the matured gonadal axis happening at any age (Young, Goy et al. 1964). The validity of this dichotomous view of estrogen actions on the brain has been challenged in the past (Arnold and Breedlove 1985; Williams 1986) but still provides a good conceptual framework. The
following sections are an attempt to briefly describe the mechanism of estrogen actions in the brain with an emphasis on neuroprotective properties of estrogens which are of interest to us in the context of this thesis.

Neuroprotection and estrogens

Accumulating evidence over the past 30 years from animals and clinical research agree that estrogens may protect the brain against the assault of aging, stroke and neurodegeneration seen in dementia. In vivo evidence showed that estrogen enhances synaptic plasticity, dendritic spine density and connectivity (Tanapat, Hastings et al. 1999; Behl 2002; McEwen 2002; Tanapat, Hastings et al. 2005). Of importance also is the neurotrophic effects of estrogens (Toran-Allerand 2004); Estrogens have profound influence on the activity of other growth factors such as brain-derived neurotrophic factors (BDNF) and nerve growth factor (NGF) (Toran-Allerand 2004; Scharfman and MacLusky 2006). Recently, BDNF levels were measured in cycling and postmenopausal women. The results demonstrated that BDNF levels are influenced by both endogenous and exogenous estrogen levels with higher levels during higher estrogen levels across the menstrual cycle, lower levels following menopause and restored levels in postmenopausal women using HT. Moreover, they observed an age-related decline in BDNF levels and a negative correlation with years since menopause (Begliuomini, Casarosa et al. 2007).

Estrogens also promote neurogenesis in the rat dentate gyrus of the HC (Tanapat, Hastings et al. 1999; Gould, Tanapat et al. 2000). Recently, it has been shown that this effect was also dose and time-dependent (Tanapat, Hastings et al. 2005). Estrogen was increasing cell proliferation only if a moderate dose was applied, not at a low or a high dose. Moreover, an acute treatment with the right moderate dose was not stimulating neurogenesis in animals that were in a prolonged state of estrogen deficiency (Tanapat, Hastings et al. 2005). This has important implication if we believe that there is an estrogen critical window of action.

Moreover, in vitro evidence suggests that estrogen might act as a chemical shield by protecting neurons against a wide range of acute and age-related insults such as amyloid-β toxicity and glutamate excitotoxicity in the hippocampus particularly (Behl 2002; McEwen 2002; Bhavnani, Berco et al. 2003). Bhavnani et al (2003) demonstrated that the different compounds in the conjugated equine estrogens
(CEE) preparation, often use as HT, were all neuroprotective against glutamate-induced neurotoxicity in hippocampal cultured cells but in a dose-dependent manner and with different potencies (for extensive reviews see Wise, Dubal et al. 2001; Behl 2002; McEwen 2002; Foster 2005).

**Estrogens and neurotransmitters**

Estrogen effects on the cholinergic, serotoninergic, and dopaminergic systems may be helpful in explaining observed sex difference in cognitive function, menstrual cycle variability in mood and cognition, and HT modulation following menopause of several cerebral functions, affective state, reproductive behavior, and motor functions. In vivo and in vitro studies have provided good evidence of the interaction estrogens-neurotransmitters as well as sex differences (organizational and activational effect) in neurotransmitters regulation (McEwen and Alves 1999).

Studies on estrogen-induced enhancement of the cholinergic enzymes by Luine (Luine 1985) for example were among the first to point toward non-reproductive actions of estrogens. Choline acetyltransferase (ChAT) is an enzyme involved in the synthesis of acetylcholine, Estrous cycle and ovariectomy/estrogen add-back studies revealed an induction of ChAT in the basal forebrain of female rats only after a short period of time and in two of its projections area namely the cortex and hippocampus after a longer delay.

In humans, it has been recently reported that long-term HT can enhance cholinergic function in postmenopausal women as measured by growth hormone (GH) responses to pyridostigmine. Interestingly, the authors state that the effect might be related to duration of estrogen use (van Amelsvoort, Murphy et al. 2003). These results may be of particular relevance for cognitive function in postmenopausal women or women suffering from Alzheimer disease (AD) since some cognitive impairments seen in AD are secondary to cholinergic deficits (McEwen and Alves 1999).

Several evidence suggest that estrogen might be implicated in modulation of women's affective state. Animal studies investigating the interactions between estrogens and serotonin, a neurotransmitter involved in mood regulation and often a prime target for pharmacological treatment of depression, revealed selective estrogen-induced changes in serotonin transmission, binding and metabolism in
cerebral regions implicated in affect regulation such as the amygdala. The interaction between estrogens and serotonin are well characterized with regard to reproductive behaviors and ovulatory regulation (Morello and Taleisnik 1985; Speroff, Glass et al. 1999; Speroff and Fritz 2005) but needs further investigation for their implication in mood regulation.

In general, studies on estrogens replacement in ovariectomized animals demonstrate that administration of estrogen affect serotonin neurons as well as its afferent neurons and target neurons (for detailed review see(Bethea, Lu et al. 2002; Amin, Canli et al. 2005)). Moreover, it has been shown that estrogens selectively increased serotonin receptors density in brain regions containing estrogen receptors such as the hypothalamus, the preoptic area, the hippocampus and the amygdala (Biegon and McEwen 1982; Biegon, Reches et al. 1983).

In women, it has recently been shown that compared to young reproductive women, postmenopausal women not using HT have a blunted serotonin response measured by either the serotonin agonist meta-chlorophenylpiperazine (m-CPP) (Halbreich, Rojansky et al. 1995) or the prolactin responses to the specific 5-HT releasing and re-uptake inhibiting agent, d-fenfluramine (van Amelsvoort, Abel et al. 2001). Estrogen therapy had a positive effect on 5-HT tone since both acute (Best, Rees et al. 1992; Halbreich, Rojansky et al. 1995) and long estrogen treatment (HT) duration (van Amelsvoort, Abel et al. 2001) were associated with increased serotonin responsivity. Thus, estrogen may modulate age-related changes in 5-HT tone. The modulation of serotonin by estrogens might be relevant to hormone-linked affective changes, hormone-linked emotional disturbances in women as well as cognition.

Finally, both human and animal literature indicates that there are numerous interactions between estrogens and dopamine regulation. Estrogen actions are both pro and anti-dopaminergic depending on the dose, duration of treatment, interval between steroid administration, and behavioural end-point, and the system recruited (nigrostriatal and mesolimbic dopaminergic systems) (Di Paolo 1994; McEwen and Alves 1999). Sex difference and gonadal modulation of dopaminergic function in striatum and nucleus accumbens have been observed (Thompson and Moss 1994; Becker 1999). Estrogens seem to enhance both the acute behavioural and neurochemical responses and the sensitization effect to psychomotor stimulants (amphetamine-apomorphine) in female rats compared to males. Becker (Becker
1999) even proposed a model for the mechanisms through which estrogen impact dopaminergic functions. It is suggested that estrogens induce rapid changes in neuronal excitability by acting on membrane receptors located on striatal neurons, dopamine terminals, and presynaptic dopamine terminals by down regulating D2 dopamine autoreceptors resulting in enhance dopamine release.

Dopamine function in the context of estrogens modulation can be studied using growth-hormone response to apomorphine in humans. Study results revealed that postmenopausal women using ET for a long duration have enhance GH responses, suggesting increased dopaminergic tone as compared to never users (Craig, Cutter et al. 2004) and minor effect with acute ET administration possibly because of variables such as prior endogenous and exogenous estrogen exposure (Best, Rees et al. 1992). These results are important with regard to sex difference susceptibility to addiction to psychomotor stimulants, neuropsychiatric disorders such as Parkinson’s disease, and reproductive behavior such as pacing behavior seen in female animals.

**Estrogens and cognition**

Another example of estrogen affecting the brain is its effects on cognitive functions. Studies in young cycling women and post-menopausal women have shown that memory performance may varies as a function of fluctuating endogenous levels and/or exogenous estrogen administration.

**Menstrual cycle and cognition**

Based on the neurobiological findings described earlier, the rationale underlying these studies is the following: since gonadal hormone secretion is known to change in a predictable manner across the menstrual cycle, corresponding changes in cognitive functioning is predicted. Thus, the design of these studies typically consist of a within design with cognitive assessments occurring at late luteal/early follicular phase (low estrogen levels) and another one close to ovulation, late follicular/early luteal phase (high estrogen levels). An early study (Broverman, Vogel et al. 1981) indicated a specific beneficial effect of higher endogenous estrogen levels on a task recruiting both attentional and working memory functions. Studies from Hampson’s laboratory showed a differential effect across the menstrual cycle.
on several articulatory, verbal, motor and perceptual tests that typically yield sex differences in performance, showing that estrogen levels could be partially responsible for these sex differences (Hampson and Kimura 1988; Hampson 1990; Hampson 1990). Recently, a study investigating these effects demonstrated that during the period of high endogenous estrogen levels, performance on a verbal working memory task was improved but spatial abilities did not differ (Rosenberg and Park 2002). Another study tried to broaden the investigation by examining sexually dimorphic abilities and different types of memory. Their results suggest that estrogens may be involved in conceptual implicit memory. They also replicated the specific effect of higher estrogen levels on verbal fluency (no effect on mental rotations) (Maki, Rich et al. 2002).

Taken together, these results suggest a positive effect of higher endogenous estrogen levels on different memory tasks usually yielding to sex difference such as declarative memory tasks. However, as previously described, the menstrual cycle consists of a well organized orchestra of hormones that fluctuate in concert and not just a solo performance performed by estrogens. Probably the major confounding hormone at this point is fluctuating progesterone which, in it self, prevent us from driving definite conclusions about the sole effect of estrogens on cognition in young cycling women. Moreover, the ongoing hormonal concert give rise to methodological issues and render this type of studies difficult to accomplish and compare. However, by carefully monitoring the menstrual cycle, it is possible to study cognitive functions in relation to gonadal hormone levels.

Pregnancy and cognition

Pregnancy represent a time in women’s life when gonadal hormones are at a supraphysiological levels and thus a unique opportunity to investigate the effects of increasingly high levels followed by a rapid decline of these hormones on cognitive functions. However, both designing and interpreting the results of this type of study is a challenge. The uniqueness of this event in each woman plus the tremendous psychological and physiological changes in different hormonal systems occurring in this short period of time prevent researchers to derive clear conclusions about the effect of one hormone, on one function, namely estrogens on cognitive functions.
Despite these obvious difficulties, studies investigating such a rich period should not be put aside.

Since these study designs are particularly heavy, only few studies investigating the effect of pregnancy on cognitive function exist. Interestingly, the results of these studies raise the question whether too much estrogen is like not enough. It was found that memory performance of pregnant women are poorer compared to controls and this at any pregnancy stages (Sharp, Brindle et al. 1993; de Groot, Hornstra et al. 2003; de Groot, Vuurman et al. 2006). Compared to themselves they were more impaired in aspects of verbal memory prior delivery (Buckwalter, Stanczyk et al. 1999) and demonstrated a significant decline on a story recall task from the second to the third trimester (Keenan, Yaldoo et al. 1998). Other studies found that pregnant women report significantly more daily forgetfulness and memory impairments then non-pregnant ones but were not able to confirm it with objectives measures (Casey, Huntsdale et al. 1999; Janes, Casey et al. 1999; Crawley, Dennison et al. 2003).

Overall, a large amount of endogenous estrogens in pregnancy seems to be linked to a decrease in some memory functions. However, due to methodological flaws such as the cross-sectional nature of these studies, small sample size, accurate measures of gonadal hormones and the rise of other hormones such as cortisol, the effects of estrogens on cognition in pregnancy remain equivocal. Despite these considerations, some of these early results pointed toward an inverted-U shape function that is currently under investigation in the field of exogenous estrogen exposure, namely HT effects on the brain and cognition.

**HT and cognition**

Investigating post-menopausal women with and without HT offers the possibility to assess the effects of estrogens without the confounding effect of the cyclic process of estrogens, or the effects of other ovarian hormones. However, randomized trials, cross-sectional studies as well as epidemiological research have led to mixed results and discrepancies. An overall beneficial effect of estrogens on cognitive function has been found in some studies (Kimura, 1995; Rice et al., 2000) while some laboratories suggest selective beneficial effects of HT on visual memory (Hogervorst, Boshuisen et al. 1999; Duka, Tasker et al. 2000; Resnick and Maki
2001; Smith, Giordani et al. 2001; Resnick, Maki et al. 2006), others on verbal memory (Sherwin 1988; Sherwin and Tulandi 1996; Wolf, Kudielka et al. 1999; Drake, Henderson et al. 2000; Maki and Resnick 2000; Maki, Zonderman et al. 2001; Stephens, Bristow et al. 2006; Stephens, Pachana et al. 2006; Yonker, Adolfsson et al. 2006), and others on working memory (Shaywitz, Shaywitz et al. 1999; Duff and Hampson 2000; Janowsky, Chavez et al. 2000; Keenan, Ezzat et al. 2001; Joffe, Hall et al. 2006; Krug, Born et al. 2006; Wegesin and Stern 2007). Furthermore, some researchers did not find any significant cognitive changes linked to estrogens (Barrett-Connor and Kritz-Silverstein 1993; Binder, Schechtman et al. 2001; Grady, Yaffe et al. 2002; Galen Buckwalter, Crooks et al. 2004; King, Travers et al. 2004; Alhola, Polo-Kantola et al. 2006; Almeida, Lautenschlager et al. 2006; Grigorova and Sherwin 2006; Kurt, Bekci et al. 2006; Low, Anstey et al. 2006; Yaffe, Vittinghoff et al. 2006; LeBlanc, Neiss et al. 2007).

One of the major confounding variables in most of these studies is the inclusion of women taking a combination of estrogen and progesterone (EPT) in the experimental group. The progestogen effects on cognitive function are unclear, but clearly introduce another source of variability. Furthermore, the duration of the HT treatment, the number of years since menopause, and the influence of endogenous estrogens are never taken into account. Cognitive tasks differ between laboratories and are often difficult to compare because of the use of non-standardized tasks. Also, the use of single mental status test such as the mini-mental state examination (MMSE) may lead to over interpretation of cognitive abilities (Rice et al., 2000; Yaffe et al., 1998). Estrogens interact with serotonin receptors, and HT is known to alleviate the depressive symptoms linked to menopause; in order to control for this effect, mood needs to be assessed (McEwen and Alves 1999). Finally, current estrogen levels are rarely assessed and hence the link to cognitive function remains elusive.

For all these reasons, new results on the effects of estrogen now introduced the idea that measuring lifelong endogenous and exogenous exposure could probably account for the discrepancies and clarify the implication of estrogens on brain function later in life (Ancelin and Ritchie 2005; Dunkin, Rasgon et al. 2005). In 1999, Smith and al. developed a global index of estrogen exposure accounting for some markers known to influence endogenous and exogenous estrogen concentration
and duration of exposure. After performing a factor analysis, they demonstrated that the total index score was positively related to two verbal factors, one attentional and one global in nature. Interestingly, the association between cumulative estrogen exposure and cognition remained significant even after controlling for current estrogen use. Measures of endogenous estrogen exposure prior menopause using reproductive period (age menopause-age menarche) and/or markers of greater endogenous exposure such as nulliparity and late menopause were linked to better cognitive functioning later in life (McLay, Maki et al. 2003; Dunkin, Rasgon et al. 2005; Rasgon, Magnusson et al. 2005). Regarding exogenous exposure, variables such as HT duration and timing of HT initiation have been investigated, demonstrating that early initiation could be beneficial whereas initiation after a long period following menopause could lead to detrimental effects on certain cognitive functions (MacLennan, Henderson et al. 2006). Overall, estrogen exposure across life span most probably acts as a modulator in memory function in postmenopausal women.

**Estrogens and neuroimaging**

Structurally, the neuroprotective effect of estrogens has not been clearly demonstrated. Studies investigating the differential effect of sex on the known age-related gray matter loss have brought interesting results about the estrogen potential to protect brain integrity in aging. More recently, results from studies investigating the effect of HT on the brain using in-vivo techniques clarified the picture but further studies are greatly needed before making any definite conclusions.

**Aging, sex and neuroimaging**

It has been repeatedly observed that the velocity of age-related gray matter loss is greater in men than in women (Cowell, Turetsky et al. 1994; Coffey, Lucke et al. 1998; Gur, Turetsky et al. 1999; Xu, Kobayashi et al. 2000; Goldstein, Seidman et al. 2001; Pruessner, Collins et al. 2001; Raz, Gunning-Dixon et al. 2004), with some studies reporting inconclusive results (Resnick, Pham et al. 2003; Lemaitre, Crivello et al. 2005; Smith, Chebrolu et al. 2006; McHugh, Saykin et al. 2007). Interestingly, the studies reporting no difference often assessed older individuals (mean age of 70 and more), questioning the linearity of the relationship between age and sex-related
brain volume decline difference. One possible explanation that has repeatedly been put forward in order to explain the differential effect of sex on the pace of brain aging is the implication of gonadal steroids such as estrogens. Based on animal research, it has recently been hypothesized that the sexual difference might be linked to the known sex difference in estrogen exposure during brain development (Goldstein, Seidman et al. 2001). It has also been attributed to lifelong or current differences in circulating estrogen levels (Cowell, Turetsky et al. 1994; Coffey, Lucke et al. 1998; Gur, Turetsky et al. 1999; Xu, Kobayashi et al. 2000; Pruessner, Collins et al. 2001; Raz, Gunning-Dixon et al. 2004).

One cross-sectional study demonstrated that age and sex could predict volume decline in the hippocampus in early adulthood (age ranged 18-42 years). Interestingly, men tend to show a linear atrophy starting after the 3rd decade of life whereas volume in women of this age range remains constant (Pruessner, Collins et al. 2001). However, Jack & al. (1998) demonstrated using a longitudinal design that hippocampal atrophy observed in elderly population does not show a sex-effect on the age related decline (70 to 89 years of age) (Jack, Petersen et al. 1998). The missing link between these studies could perhaps be the occurrence of menopause (see Figure 3). The rapid drop of endogenous estrogen levels following menopause and the following estrogen deprivation state could probably be responsible for the atrophy seen in older ages and the absence of sex differences, reflecting Jack’s results (Jack et al., 1998). The results of Pruessner et al. (2001) would reflect the protective effect of endogenous estrogen exposure prior menopause on a brain area involved in memory.
Figure 3: Schematic representation of the hypothetic differential effect of sex on the age-related volume decline.

However, the cross-sectional nature of most of these studies could add cohort effects which are difficult to control for. Moreover, the relevant studies are difficult to compare because of different age-ranges, different inclusion criteria, different brain regions of interest, and different analyses and techniques. Therefore, there is no consensus among studies on which regions are structurally sensible to sex difference.

Hormone therapy and neuroimaging

Information about the effects of estrogens on the brain has recently been enriched by the advent of neuroimaging techniques. Studies using magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) and magnetic resonance spectroscopy (MRS) are among the ones providing new in vivo insight about the effects of HT on cerebral structures. To this date, only few studies investigated the effects of estrogens on the brain in healthy pre and postmenopausal women.
HT and magnetic resonance imaging

While numerous studies have been examining the effects of estrogen and HT on cognitive function (for a review see (Zec and Trivedi 2002)), few studies have focused on the influence of either ET or HT on brain integrity in aging using anatomical magnetic resonance imaging (MRI). Eberling and al.(2003) found that post-menopausal women using ET had larger right hippocampi compared to non-users (Eberling, Wu et al. 2003). Also, there is evidence from a longitudinal study of HT-related sparing of age-related cortical regions decline (Raz, Rodrigue et al. 2004). Moreover, it has been shown that women using ET showed a selective sparing of brain tissue decline with aging as a function of treatment duration, with grey matter preserved in prefrontal, parietal, and temporal regions and white matter in medial temporal lobe regions (Erickson, Colcombe et al. 2005).

Recently, Erickson and colleagues (2007) added to the debate by demonstrating a possible interaction between HT duration and physical fitness on brain volume measured through voxel-based morphometry (VBM) (Erickson, Colcombe et al. 2007). Their results seemed to suggest that long-term HT (> 10 yrs) use is increasingly detrimental, but can be offset with high levels of physical fitness. Along the same line, another study compared brain volume between current ET users, past HT users and never users and found that past users had the largest gray matter volume (Boccardi, Ghidoni et al. 2006). Interestingly, in this study the mean treatment duration was 7.5 years for current and past users suggesting that past users were benefiting from a long enough treatment. These findings argue in favor of a beneficial long-term effect of HT on the brain even after treatment cessation as seen in the past users group. Taken together, these findings are in line with those obtained in the cognitive domain and suggest that ET may indeed have positive long-term effects on brain morphology if a specific duration threshold is not exceeded.

HT and magnetic resonance spectroscopy

Using proton magnetic resonance spectroscopy (MRS), a technique that can measures ratios and concentrations of different compounds such as N-acetyl-L-aspartate compounds (NA; a measure of neuronal density and integrity), myo-inositol (MI; a putative glial marker whose levels reflect glial content or activity) creatinine and phosphocreatinine (marker of phosphate metabolism) and choline containing
compounds (Cho; an indication of membrane metabolism), revealed neuroprotective effect of HT.

Two recent studies investigated the effect of HT on brain metabolites (Robertson, van Amelsvoort et al. 2001; Ernst, Chang et al. 2002). Robertson et al. demonstrated that postmenopausal women not using HT had significantly more Cho compared to long-term ET users and young cycling women in the parietal lobe and hippocampus demonstrating that long-term estrogen use may modulate neuronal/glial membrane turnover (Robertson, van Amelsvoort et al. 2001). The other study assessed all four previously mentioned compounds in the frontal white matter, basal ganglia, and hippocampus of healthy postmenopausal using HT or tamoxifen or no medication. They found that women who took tamoxifen or HT had statistically significantly lower levels of MI than did non users arguing in favour of a beneficial modulation of brain aging since normal aging is associated with increase in cerebral concentration of MI. However, they failed to replicate the Cho findings since they did not find any group difference for the other compounds.

**HT and neurotransmitters based neuroimaging techniques: PET and SPECT**

Recently, interesting studies emerged using different imaging techniques in order to investigate the interactions between estrogens and neurotransmitters systems.

Studies examining the effect of long-term postmenopausal HT on brain concentrations of cholinergic synaptic terminals in postmenopausal women using single photon emission computed tomography (SPECT) revealed that duration of HT and age at menopause were key elements to characterize the interactions between the cholinergic system and estrogens later in life (Smith, Minoshima et al. 2001). In 2007, another SPECT study was published, investigating the effect of long-term ET on brain cholinergic muscarinic receptors. Their results showed that compared to young cycling women, postmenopausal women had significantly lower muscarinic receptor density with ET users having higher muscarinic receptor density than never-users (Norbury, Travis et al. 2007). These results suggest that HT and markers of estrogen exposure may influence the survival or plasticity of cholinergic neurons in postmenopausal women.

Serotonin and estrogens interaction have also been investigated using positron emission tomography (PET) and study results revealed that short-term HT increases
5-HT(2A) receptor binding in prefrontal regions (Moses, Drevets et al. 2000; Kugaya, Epperson et al. 2003; Moses-Kolko, Berga et al. 2003).

Furthermore, only one neuroimaging pilot study exists so far on the interactions of estrogen and the dopamine system. It has been shown in the past using PET, that there is an age-related decline in D2 receptor density and that this decline is more pronounced in women (Wong, Wagner et al. 1984; Wong, Broussolle et al. 1988). Gardiner et al. (2004) demonstrated in her SPECT pilot study that short-term use of HT is associated with a modest increase in dopamine transporter (DAT) in the anterior putamen and not the caudate nucleus (Gardiner, Morrison et al. 2004).

Overall, we believe that evidence emerging from in vitro, in vivo as well as different field in human research converge toward the implications of indices of lifelong estrogen exposure in order to fully understand the extent of estrogen effects on the brain.
**General goal of the thesis**

New findings allow us to suggest that lifelong exposure to endogenous estrogens could be a reliable predictor of the beneficial or detrimental effect of exogenous estrogens on cognition later in life, thus explaining why some women on HT show beneficial effects while others show detrimental effects of exogenous estrogen administration after menopause.

The current studies have been designed to investigate the effect of estrogens on the brain during aging from a new perspective by introducing new measures.

First, a questionnaire assessing indices of lifelong estrogen exposure was developed and provided us with several important variables of estrogen exposure. The aim of this original thesis was first to assess the reliability of indices of lifelong endogenous and exogenous estrogen exposure.

Secondly, we recruited postmenopausal women and men of the same age group to assess the effect of estrogens on the brain using magnetic resonance imaging. Since we wanted to isolate as much as possible the effect of estrogens on the brain, our group of postmenopausal women currently using HT were unopposed ET users only. Finally, volumetric analyses of regions of interest in the brain (hippocampus and amygdala) as well as global automated analysis using voxel-based morphometry (VBM) were performed on the magnetic resonance images to assess whether current ET use as well as estrogen exposure throughout life can predict age-related structural changes in the aging brain.
Chapter 2:
Measuring Indices of Lifelong Estrogen Exposure: Self-Report Reliability

Catherine Lord, Annie Duchesne, M.Sc., Jens C Pruessner, Ph.D.,
& Sonia J Lupien, Ph.D.

Under review in «Climacteric» at the moment of submitting this thesis
Preface to Chapter 2

Since the publication of the Women Health Initiative (WHI) study results assessing the risks and benefits of hormone therapy, efforts have been made to better understand the lifelong effects of gonadal hormones on women's health, particularly on the brain and cognition. While scientists studying breast cancer and cardiovascular diseases have identified a number of reproduction-related events as part of the etiology of these conditions, it is only recently that neuroscientists started to explore the potential of these endogenous events in modulating the effects of estrogens on the brain and cognition.

In order to take these recent findings into account, we developed a questionnaire assessing lifelong indices of endogenous and exogenous estrogen exposure. Given the self-report nature of this measure, we decided to assess if self-reported indices such as the one in our in-house questionnaire are reliable. Reliability measures allow us to conclude about the reproducibility across time of self-reported lifelong estrogen exposure indices in this particular case.

Fifty six healthy postmenopausal women were invited to complete the questionnaire at two occasions within a four year interval. Indices were extracted and answers from time 1 were correlated to the one provided at time 2. Since age has been reported to introduce a recall bias, we assessed if older women are less concordant with themselves over time when compared to younger women. Interestingly, we found that most lifelong estrogen exposure indices were reliable. An effect of age on the recall of these indices was also observed, with the older being less reliable. However, age at menopause and age at hormone therapy initiation led to weaker correlations across time of measurements driven by the lack of reliability of the older women. Due to the importance of these variables, gathering information from physician/pharmacy records could improve the reliability and validity of these indices.

More importantly for the current thesis, these results allow us to ascertain that the indirect and self-reported measures used by us and even others were sufficiently reliable to pursue further investigations about the effect of lifelong estrogen on the brain in aging using self-reported indices.
Waiver from co-authors

As co-authors of the manuscript «Measuring Indices of Lifelong Estrogen Exposure: Self-Report Reliability.», we give to Catherine Lord the authorization to include this unpublished manuscript in the present dissertation as a partial fulfillment of her PhD degree in Neuroscience at McGill University.

Annie Duchesne

Jens C. Pruessner

Sonia J. Lupien
Manuscript:

Measuring Indices of Lifelong Estrogen Exposure: Self-Report Reliability

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Running title: Self report reliability of estrogen exposure

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Abstract:

Objective

Since the publication of the results of the Women Health Initiative, efforts have been made to better understand the lifelong effects of gonadal hormone secretion on women's health, particularly on cognition and the brain. Based on the growing body of evidence, we decided to assess the reproducibility of self-reported lifelong estrogen exposure indices by measuring self-agreement.

Design

We first developed a questionnaire assessing lifelong indices of endogenous and exogenous estrogen exposure. The questionnaire was filled by 36 healthy postmenopausal women twice within a four year interval. We tested reliability using Pearson's and Kappa correlation coefficients. We also assessed if older women are less concordant with themselves overtime when compared to younger women.

Results

In this study, we demonstrated strong significant correlations for most lifelong estrogen exposure indices as well as an effect of age on the recall of these indices. However, age at menopause and age at hormone therapy initiation led to weak correlations across time of measurements and to no relation between Time 1 and Time 2 when looking at the older women.

Conclusions

Due to the important nature of these variables we suggest gathering information from physician/pharmacy records to reduce systemic error even if the reliability of most of the self-reported lifelong estrogen indices is good. Other variables such as hormone therapy brand and dosage, fitness level, weight, and neonatal estrogen exposure could also be beneficial for the understanding of lifelong estrogen exposure.

Keywords (6 or less): estrogen exposure, reliability, reproducibility, age at menopause, hormone therapy, reproductive history
Introduction

Since the publication of the Women Health Initiative (WHI) study results assessing the risks and benefits of hormone therapy, efforts have been made to better understand the lifelong effects of gonadal hormones on women's health, particularly on the brain and cognition. While scientists studying breast cancer and cardiovascular diseases have identified a number of reproduction-related events as part of the etiology of these conditions, it is only recently that neuroscientists started to explore the potential of these endogenous events in modulating the effects of estrogens on the brain and cognition. While a lot of attention is devoted to understanding the impact of hormone therapy (HT) on cognitive function (Zec and Trivedi 2002), a recent hypothesis has been put forward that suggests a possible impact of lifelong exposure to endogenous estrogens in modulating the effect of HT on cognitive function (Ancelin and Ritchie 2005; Sherwin 2007).

An early study attempting to link lifelong estrogen exposure to cognitive function in postmenopausal women demonstrated that some reproductive events as well as an index pooling lifelong endogenous and exogenous estrogen exposure markers was significantly related to cognitive performance (Smith, McCleary et al. 1999). More recently, variables such as timing of HT initiation have been investigated, demonstrating that early initiation could be beneficial whereas initiation after a long period following menopause might lead to detrimental effects on certain cognitive functions (MacLennan, Henderson et al. 2006). Measuring the endogenous estrogen exposure prior to menopause using, for example, the duration of the reproductive period (by subtracting the age of menarche from the age at menopause), or markers of greater endogenous exposure such as nulliparity and late menopause were linked to better cognitive functioning later in life (McLay, Maki et al. 2003; Dunkin, Rasgon et al. 2005; Rasgon, Magnusson et al. 2005). Furthermore, duration of HT is now taken into account but is leading to mixed results so far (Schmidt, Fazekas et al. 1996; Erickson, Colcombe et al. 2005; MacLennan, Henderson et al. 2006; Erickson, Colcombe et al. 2007; Lord, Buss et al. in press). Apart from these studies, theoretical papers have been published arguing in favour of further assessment of a woman reproductive history in order to reconcile the HT findings and better characterize the effect of estrogens on the brain and cognition (Ancelin and...
Ritchie 2005; Maki 2006; Maki 2006; Resnick, Maki et al. 2006; Genazzani, Pluchino et al. 2007; Sherwin 2007). All of these studies underscore that there is a continuous and controversial debate on whether HT has beneficial effects on the brain and cognition.

In order to take these recent findings into account, we developed a questionnaire assessing lifelong indices of endogenous and exogenous estrogen exposure. Given the self-report nature of this measure, the first factor we assessed was the reliability of our measure when administered at two time points. Consequently, the goal of the present study was to assess the reproducibility of self-reported lifelong estrogen exposure indices by measuring self-agreement within a four year interval in the same individuals who filled out our questionnaire. Since age has been reported to introduce a recall bias (Colditz, Stampfer et al. 1987; den Tonkelaar 1997; Hahn, Eaker et al. 1997), we assessed if older women are less concordant with themselves over time when compared to younger women.

Methods

Participants

Subjects were 36 healthy postmenopausal women from 50 to 79 years of age at the first assessment. Women were recruited from ongoing aging studies from our laboratory. The study was approved by the Douglas Mental Health University Institute Ethic committee and all participants provided informed written consent. Participants were asked to provide information about their age, education, income and general health, psychiatric and neurological conditions along with completing our in-house questionnaire assessing retrospectively different lifelong estrogen exposure indices. The questionnaire assesses different markers that are known to affect endogenous and exogenous concentration and duration of estrogen exposure during a woman’s life. The questionnaire is divided into 6 sections. The first section is about perinatal estrogen exposure. The second section is on menstrual cycle history: age at menarche, amenorrhea, and contraceptive pill usage. The third section is on motherhood, pregnancies, miscarriage and breast-feeding. The fourth section is assessing the menopausal status and the fifth section is hormone therapy history. The last section assesses information about possible confounds such as weight, height (in
order to calculate the body mass index (BMI)), usage of cigarette and subjective fitness levels throughout life. The questionnaire can be found in the appendix. The questionnaire was either mailed to the participants or filled out during a supervised appointment, depending on the underlying study design. Data were entered and coded by two different persons to prevent errors. Data were first acquired in 2002-2003 (n=36, age range from 50-79) and the follow-up took place in 2006-2007 (n=36, age range from 54-83). The test-retest nature of the study allowed us to assess the reliability of the self-reported variables. Agreement between participant’s responses at time 1 and time 2 was evaluated for age at menarche, use of contraceptive pill, duration of use of contraceptive pill, number of pregnancies, breast-feeding, having or not experienced pregnancies that were not brought to term, number of pregnancies that were not brought to term, type of menopause, age at menopause (age at last menstruation for women who experienced a natural menopause, age at ovarectomy/hysterectomy for surgically menopausal women and age at HT initiation for women who had an hysterectomy alone), HT use, age at HT initiation, age at HT cessation and duration of HT.

**Statistical analysis**

To determine reproducibility, we used Pearson’s correlation coefficients to compare the first reported continuous variables (Time 1) to the recalled variables at follow-up (Time 2). For discontinuous variables we used the Kappa coefficient. Finally, in order to assess the effect of age, we divided our subject in two age groups (n\text{younger} = 21, 50 to 65; n\text{older} = 15, 66 to 79 years of age at time 1) and compared the respective correlation coefficients for each indices of estrogen exposure.

**Results**

Table 1 shows the different mean and standard deviations or proportions of the previously named variables at Time 1 and Time 2, along with the appropriate correlation coefficients. Number of pregnancies that were not brought to term, HT use (yes/no), pregnancies that were not brought to term (yes/no), number of pregnancies, type of menopause, age at menarche, use of contraceptive pill, age at
HT cessation, duration of HT demonstrated in this order a strong reproducibility over time (all r or k above 0.84, coefficient of determination ($r^2$) ranging from 0.71 to 0.96). Duration of use of contraceptive pill, breast-feeding of the first born (yes/no), age at menopause shows moderate correlations (0.77, 0.72 and 0.68 respectively, coefficient of determination ($r^2$) ranging from 0.46 to 0.59) whereas age at HT initiation demonstrated a weak correlation of 0.43 ($r^2=0.18$).

Table 2 presents the appropriate coefficients for each age group. When the group is separated as a function of age ($n_{\text{younger}}=21$ and $n_{\text{older}}=15$), we can see that age at menarche, use of contraceptive pill (yes/no), pregnancies brought or not to term and their number, HT use (yes/no), and duration of HT remained strongly associated (all r or k above 0.8, coefficient of determination ($r^2$) ranging from 0.64 to 0.96) in both age groups. Duration of use of contraceptive pill, type of menopause, age at menopause, age at HT initiation and cessation were negatively affected by age, demonstrating stronger correlations in the younger group. Duration of use of contraceptive pill and age at menopause became strongly correlated (0.83, 0.95 respectively) in the younger individuals whereas age at menopause, age at HT initiation and cessation did not even reach significance in the older group (all $p>0.05$).

A power analysis was performed using the program «Power and Precision 2.1» (Biostat, Englewood, USA) which employs the Fisher's Z transformation revealed that for an expected effect size of 0.50 with significance level set at 0.05, one tailed, we had 92 % chance to yield statistically significant results with our main sample size of 36. Once this main group was divided in two, we had 82 % ($n_{\text{younger}}=21$) and 68% ($n_{\text{older}}=15$) chance to reject the null hypothesis. The selected effect size was defined as the smallest effect of substantive significance.

**Discussion**

In this study, we demonstrate significant strong correlations for most key variables assessing indices of lifelong estrogen exposure recalled by women on two occasions as well as an effect of age on the recall of these indices. Age at menopause and age at HT initiation were the two variables leading to weak correlations across
time of measurements and to no relation between Time 1 and Time 2 when looking at the group of older women (over 65 years of age).

Age at menarche is a central piece of the puzzle when assessing the effect of endogenous estrogen exposure along with age at menopause. In this study, age at menarche revealed a strong reliability across time in both age groups, whereas other studies reported a moderate agreement when the validity of self-reported age at menarche later in life was assessed (Livson and Mc 1962; Damon and Bajema 1974; Bean, Leeper et al. 1979; Casey, Dwyer et al. 1991; Cooper, Blell et al. 2006). One study categorized age at menarche into early, normal and late and observed an improved agreement (Cooper, Blell et al. 2006).

Menopausal status, another crucial element when assessing this lifelong endogenous estrogen exposure, needs as much precision as possible to accurately code the data. Other studies have investigated the reliability of self-reported age at menopause and are in line with our low agreement and age recall bias findings. These studies found that age of, or years since menopause are significantly associated with the difference in recall. Further, women who had undergone surgical menopause seem to be more accurate than women reporting a natural menopause (Colditz, Stampfer et al. 1987; den Tonkelaar 1997; Hahn, Eaker et al. 1997). Due to our small sample size, we were not able to assess the difference between surgical or natural menopause women; however we can say that 9 out of 10 women reporting a surgical menopause reported the same age at menopause. These results remind us about the complexity of this measure and the need for precision when retrieving the information and this, even more with older adults.

Overall, in the current study, indices of exogenous estrogen exposure led to a high degree of reproducibility over time with the exception of timing of HT initiation and again this weak agreement was even more pronounced in older women. Few studies have investigated the reliability and validity of self recall use of HT. Most studies compared self-report with physician/pharmacy records and founded a moderate to good agreement (Goodman, Nomura et al. 1990; Jain, Rohan et al. 1999; Paganini-Hill and Clark 2007) for treatment use and duration, similar to what we report.

However, even if overall these indices seem reliable, other critical factors such as age at menopause and age at HT initiation and cessation need improvement
and greater agreement should be reached in younger but most importantly in older postmenopausal women. Gathering information from physician/pharmacy records when accessible might reduce systematic errors and come close to the true value for age at menopause and HT usage. Type and age at menopause could also be ensured by this method, increasing reliability for natural menopausal women as well as for surgically induced menopause assuring that women who report it also had an oophorectomy along with a hysterectomy. Getting information from other sources would also allow us to obtain information about HT brand and dosage which has been proven to be difficult to recall. Another way to reduce error could be to include in study designs an early assessment self-agreement between two self-reported measurements, like in the present study, and identify less reliable women at an early stage in study process. These subjects could then be flagged or even discarded and replaced by equivalent subjects if possible, or invited to provide more information in a more thorough interview if medical records are not available. Another variable worth assessing in order to increase reliability for HT history is compliance behaviour to the treatment either by questionnaire, diary or during an interview.

Once all the factors influencing the reproducibility, and consequently the possible source of errors, are identified and controlled for by the different strategies outlined above, it is then important to evaluate the best way to analyse the resulting information. The question remains if several indices would provide a better and more precise explanation than reducing variability (and thus error) by clustering the data into an index of lifelong estrogen exposure. Given that the effects of estrogens on the brain and cognition have not been fully elucidated, we believe that future studies should concentrate their efforts on first investigating the potential indices and establish if there is any relationship between them and cognition because they are rich in important. For example, details such as age at HT initiation would be lost in an index of exogenous estrogen exposure accounting for the duration only or details linked to timing of reproductive events. Once the relationship with precise indices is investigated, we could then create a more reliable and valid index of estrogen exposure and assess causation with higher statistical power.

From our point of view, additional indices should be added in order to better understand lifelong estrogen exposure and we have incorporated these in the latest revised version of the present questionnaire (see Appendix). Factors such as lifelong
body mass index and fitness levels would be important to consider since they are known to influence endogenous estrogen levels and/or actions. Prenatal estrogen exposure would also be important to consider since during this period, the foetus is exposed to high levels of gonadal hormones. In the breast cancer research field, increased prenatal estrogen exposure seems to be associated with increasing risk for the disease (Potischman, Gail et al. 1999). Some indices like birth weight and size and birth order reflect variations in prenatal estrogen exposure and are associated with breast cancer (Hsieh, Trichopoulos et al. 1990; Vatten, Nilsen et al. 2005). Another indice, the ratio of 2\textsuperscript{nd} to 4\textsuperscript{th} finger digit lengths, has also been associated with variation in foetal exposure to gonadal hormones. While the ratio would be negatively correlated with foetal testosterone, it would be positively associated with estradiol (Lutchmaya, Baron-Cohen et al. 2004). A number of studies have been published recently demonstrating the relationship between cognition and early life gonadal hormone variations. Animal studies have shown that sexually dimorphic cognitive functions are due to differential exposure to estrogens during the perinatal period (Williams and Meek 1991). In humans, differences in cognitive performance have also been found in relation to this indirect measure of perinatal gonadal hormone exposure (Burton, Henninger et al. 2005).

This study suffers from important limitations such as the small sample size affecting the statistical power and limiting the generalisation of these results. However, we believe that this study supports the use of most self-reported estrogen exposure variables but also raises important theoretical as well as measurement issues that need to be addressed more thoroughly in the near future. Also, even if self-reported retrospective assessment is subject to error and recall bias, it was the best procedure to use to assess reliability in the current design since the true values of the variables were not known or accessible. However, even if reliability is a necessary factor to assess, it is not a sufficient condition to conclude about the validity of the measure and future studies are needed to establish if these self-reported variables are accurate.
Conclusions

Our findings demonstrate strong significant correlations for most lifelong estrogen exposure indices recalled by postmenopausal women on two occasions as well as an effect of age on recall. Age at menopause and age at HT initiation were the two less reliable variables and were also greatly affected by age/recall interval. In order to increase reliability overall, we suggest gathering information from physician/pharmacy records or include reliability assessment in study designs to reduce systemic error. Compliance behaviour toward HT is also a variable worth assessing. Once the data is acquired, assessing the effect of multiple indices or an index of pooled indices need future assessments and should be part of the ongoing debate. Other variables such as neonatal estrogen exposure, HT brand and dosage, fitness level, and lifelong body mass index could also be beneficial for the understanding and were introduce in the latest version of the present questionnaire on indices of estrogen exposure.

Acknowledgements

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References


Table 1 Mean and standard deviation or proportion and correlation coefficients for variables of lifelong estrogen exposure at time 1 and time 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>n time 1</th>
<th>Mean &amp; SD or % time 1</th>
<th>n time 2</th>
<th>Mean &amp; SD or % time 2</th>
<th>Pearson's correlation coefficient</th>
<th>Kappa coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age menarche</td>
<td>35</td>
<td>12.8 ± 1.4</td>
<td>36</td>
<td>13.0 ± 1.5</td>
<td>0.87 **</td>
<td></td>
</tr>
<tr>
<td>Use of contraceptive pill (yes/no)</td>
<td>36</td>
<td>y = 69% n = 31%</td>
<td>34</td>
<td>y = 64% n = 31%</td>
<td>0.87 **</td>
<td></td>
</tr>
<tr>
<td>Duration of use of contraceptive pill (months)</td>
<td>25</td>
<td>75 ± 65</td>
<td>21</td>
<td>81 ± 55</td>
<td>0.77 **</td>
<td></td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>36</td>
<td>1.8 ± 1.6</td>
<td>34</td>
<td>1.9 ± 1.7</td>
<td>0.91 **</td>
<td></td>
</tr>
<tr>
<td>Breast-feeding of the first born (yes/no)</td>
<td>26</td>
<td>y = 39% n = 54%</td>
<td>26</td>
<td>y = 35% n = 58%</td>
<td>0.72 **</td>
<td></td>
</tr>
<tr>
<td>Pregnancies that were not brought to term (yes/no)</td>
<td>35</td>
<td>y = 31% n = 69%</td>
<td>36</td>
<td>y = 28% n = 72%</td>
<td>0.93 **</td>
<td></td>
</tr>
<tr>
<td>Number of pregnancies that were not brought to term</td>
<td>36</td>
<td>0.6 ± 1.1</td>
<td>36</td>
<td>0.5 ± 0.9</td>
<td>0.97 **</td>
<td></td>
</tr>
<tr>
<td>Type of menopause (Natural/Surgical)</td>
<td>36</td>
<td>N = 67% S = 33%</td>
<td>36</td>
<td>N = 72% S = 28%</td>
<td>0.87 **</td>
<td></td>
</tr>
<tr>
<td>Age at menopause</td>
<td>36</td>
<td>48.7 ± 4.5</td>
<td>36</td>
<td>48.8 ± 3.8</td>
<td>0.68 **</td>
<td></td>
</tr>
<tr>
<td>HT users (yes/no)</td>
<td>36</td>
<td>y = 75% n = 25%</td>
<td>36</td>
<td>y = 72% n = 28%</td>
<td>0.93 **</td>
<td></td>
</tr>
<tr>
<td>Age at HT initiation</td>
<td>27</td>
<td>49.1 ± 4.5</td>
<td>25</td>
<td>48.3 ± 6.1</td>
<td>0.43 *</td>
<td></td>
</tr>
<tr>
<td>Age at HT cessation</td>
<td>27</td>
<td>62.9 ± 6.8</td>
<td>26</td>
<td>61.0 ± 7.9</td>
<td>0.85 **</td>
<td></td>
</tr>
<tr>
<td>Duration of HT (years)</td>
<td>36</td>
<td>9.9 ± 8.8</td>
<td>35</td>
<td>8.9 ± 8.6</td>
<td>0.84 **</td>
<td></td>
</tr>
</tbody>
</table>

** Significant at 0.01 level
* Significant at 0.05 level
Table 2 Correlation coefficient for variables of lifelong estrogen exposure at time 1 and time 2 per age group

<table>
<thead>
<tr>
<th>Correlation coefficients</th>
<th>50-65 at time 1</th>
<th>65 and older at time 1</th>
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<tbody>
<tr>
<td></td>
<td>Pearson coefficient</td>
<td>Kappa coefficient</td>
</tr>
<tr>
<td>0.87 **</td>
<td>0.88 **</td>
<td>0.89 **</td>
</tr>
<tr>
<td>0.87 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age menarche</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.83 **</td>
<td>0.63 **</td>
</tr>
<tr>
<td>Use of contraceptive pill (yes/no)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.77 **</td>
<td>0.93 **</td>
<td>0.84 **</td>
</tr>
<tr>
<td>Duration of use of contraceptive pill (months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.91 **</td>
<td>1.0 **</td>
<td></td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.93 **</td>
<td>1.0 **</td>
<td></td>
</tr>
<tr>
<td>Pregnancies that were not brought to term (yes/no)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.97 **</td>
<td>0.99 **</td>
<td></td>
</tr>
<tr>
<td>Number of pregnancies that were not brought to term</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.87 **</td>
<td>1.0 **</td>
<td>0.67 **</td>
</tr>
<tr>
<td>Type of menopause (Natural/Surgical)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.68 **</td>
<td>0.95 **</td>
<td>0.19</td>
</tr>
<tr>
<td>Age at menopause</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.93 **</td>
<td>0.83 **</td>
<td>1.0 **</td>
</tr>
<tr>
<td>HT users (yes/no)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.43 **</td>
<td>0.63 **</td>
<td>0.08</td>
</tr>
<tr>
<td>Age at HT initiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85 **</td>
<td>0.90 **</td>
<td>0.49</td>
</tr>
<tr>
<td>Age at HT cessation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.84 **</td>
<td>0.80 **</td>
<td>0.86 **</td>
</tr>
<tr>
<td>Duration of HT (years)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Significant at 0.01 level
* Significant at 0.05 level
Annexe 1

Revised version of the questionnaire: «Indices of Estrogen Exposure»

Indices of estrogen exposure

Dale of Birth: ___________

Many studies attempt to understand the influence of estrogen on physical and mental health. In this questionnaire we focus on determining the variations of estrogen over the course of one's life. The levels of estrogen can affect the development of various conditions, including mental health, cognitive function, and physical health. Therefore, understanding how different factors influence estrogen levels is crucial for improving overall health outcomes.

1. **Reproduction**
   - Please provide information on your reproductive history:
   - Age at first menstruation?
   - Did you ever have a miscarriage? (yes/no)
   - How many miscarriages did you experience?
   - Did you ever have a stillbirth? (yes/no)
   - How many stillbirths did you experience?

2. **Menopause**
   - If you have reached menopause, please provide:
   - Age at menopause?
   - Did you undergo hormone therapy after menopause? (yes/no)
   - If yes, please specify:
     - Type of hormone therapy?
     - Duration of treatment?
     - Any side effects?

3. **Pregnancy**
   - Please provide details on your pregnancy:
   - Number of pregnancies?
   - Age at first pregnancy?
   - Age at last pregnancy?
   - Any complications during pregnancy?

4. **Birth**
   - Please provide information on your birth:
   - Weight at birth?
   - If you were premature (born before 37 weeks of gestation), (yes/no)
   - If yes, at what age were you born?

5. **Health habits**
   - Please provide information on your health habits:
   - Do you currently smoke cigarettes? (yes/no)
   - If yes, please specify your daily consumption.
   - Do you currently engage in regular exercise? (yes/no)
   - If yes, please specify the frequency and intensity of your exercise.

Thank you for your participation.
Chapter 3:
Hippocampal Volumes are Larger in Postmenopausal Women
Using Estrogen Therapy Compared to Past users, Never users and
Men: A Possible Window of Opportunity Effect

Catherine Lord, Claudia Buss Ph.D., Sonia J Lupien, Ph.D.,
& Jens C Pruessner, Ph.D.

Preface to chapter 3

We have demonstrated by a review of the literature in the introduction of the current thesis that there is considerable evidence suggesting that estrogen can have neuroprotective effects. However, recent results raised important questions regarding the conditions under which hormone therapy (HT) following menopause can be beneficial. This led to the suggestion that variables such as lifelong estrogen exposure and time of HT initiation and duration are of critical importance for the understanding of estrogen effects on brain function and structures.

The aim of the present manuscript was to investigate the potential neuroprotective effects of estrogens in aging on brain regions with high levels of estrogen receptors, namely the hippocampus (HC) and the amygdala (AG). In order to better characterize the punctual and long term effects of estrogens we tested postmenopausal women currently using unopposed ET, past HT (ET & EPT) users, never users, and men. Lifelong estrogen exposure variables were included in the analysis namely age at menses, age at menopause, and ET duration.

At this point it would be important to point out that, based on the results of the first study and the age range of our participants (50 to 74 years old), two sets of analysis were conducted. In the first manuscript, we observed that age at first menstruation and HT duration were reliable but age at menopause showed less self-agreement in women aged above 65 years old. In order to account for these results, we conducted the analysis of the current study with and without older individuals (above 65 years of age, n=10) and decided to keep the original larger sample size since the results were not affected by the removal of the older subjects.

Accordingly, the results demonstrate that women using ET had larger left and right HC volumes compared to men, and larger right HC volumes compared to past users and never users. We also found a negative correlation between ET duration and HC volume in the ET user group. The observed effects were region-specific to the HC since we did not found difference in AG volumes. These findings demonstrate a treatment duration dependent neuroprotective role of estrogen on HC volume in aging supporting the notion that estrogen exposure is an important variable to take into account.
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Hippocampal Volumes are Larger in Postmenopausal Women Using Estrogen Therapy Compared to Past users, Never users and Men: A Possible Window of Opportunity Effect

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Considerable evidence suggests that estrogen can have neuroprotective effects. However, recent results raised important questions regarding the conditions under which hormone therapy (HT) following menopause can be beneficial. It has been suggested that variables such as time of initiation and duration of HT use are of critical importance for beneficial cognitive effects to be observed. The aim of the present study was to investigate the potential neuroprotective effects of estrogens in aging on brain regions with high levels of estrogen receptors, namely the hippocampus (HC) and the amygdala (AG). In order to better characterize the punctual and long term effects of estrogens we tested postmenopausal women currently using estrogen therapy alone (ET), past HT users, never users, and men. Age at menses, age at menopause, HT duration and age were included as covariates in the analysis. Results demonstrate that women using ET had larger left and right HC volumes compared to men, and larger right HC volumes compared to past users and never users. Importantly, we found a significant negative relationship between ET duration and HC volume in this group. The observed effects were region-specific since no significant differences could be observed for the AG. In summary, these findings support a treatment duration dependent neuroprotective role of estrogen on HC volume in aging.
1. Introduction

Considerable evidence suggests that in both animal and humans, estrogen can have positive effects on cognitive performance in aging (for reviews, see [2] and [40]). Other studies however, most notably the Women's Health Initiative Memory Study, have reported adverse effects of ovarian hormones on global cognitive function [11] and [30]. These results raised important questions regarding the positive effects of estrogens on cognition and on the conditions under which hormone therapy, either estrogen therapy alone (ET) or a combination of estrogen and progesterone (hormone therapy, HT), can be beneficial.

In an attempt to address this question, a recent study conducted by MacLennan et al. [21] examined the impact of the timing of initiation and duration of HT use on cognitive function and showed that early HT initiation was of critical importance for beneficial effects to be observed, at least in certain cognitive domains. Moreover, they demonstrated a treatment duration threshold for the beneficial effects of HT on cognitive function of 11 years [21]. This is in accordance with previous results in both humans and animals [12], [13], [23] and [29].

While numerous studies have been examining the effects of estrogen and HT on cognitive function (for a review, see [40]), few studies have focused on the influence of either ET or HT on brain integrity in aging. Eberling et al. [8] found that postmenopausal women using ET had larger right hippocampi compared to non-users [8]. Also, there is evidence from a longitudinal study of HT-related sparing of age-related cortical regions decline [31]. Moreover, it has been shown that women using ET showed a selective sparing of brain tissue decline with aging as a function of treatment duration, with gray matter preserved in prefrontal, parietal, and temporal regions and white matter in medial temporal lobe regions [10]. Recently, Erickson et al. [9] added to the debate by demonstrating a possible interaction between HT duration and physical fitness on brain volume measured through voxel-based morphometry (VBM) [9]. Their results seemed to suggest that long-term HT (>10 years) use is increasingly detrimental, but can be offset with high levels of physical fitness. Along the same line, another study compared brain volume between current
ET users, past HT users and never users and found that past users had the largest gray matter volume [4]. Interestingly, in this study the mean treatment duration was 7.5 years for current and past users suggesting that past users were benefiting from a long enough treatment. These findings argue in favor of a beneficial long-term effect of HT on the brain even after treatment cessation as seen in the past users group. Taken together, these findings are in line with those obtained in the cognitive domain and suggest that ET may indeed have positive long-term effects on brain morphology if a specific duration threshold is not exceeded.

The exact mechanisms by which estrogens exert such neuroprotective effects remain unclear but likely involve interactions between various neurotransmitter systems, neurotrophic factors, and the classical nuclear estrogen receptor (ER) particularly present in areas implicated in learning and memory such as the amygdala (AG) and the hippocampus (HC) [2], [24] and [33].

The aim of the present study was to investigate the potential neuroprotective effects of estrogens on high ER density structures namely the HC and the AG in aging. Specifically, we sought to investigate the effects of the duration of ET on brain integrity. We propose that there likely exists an optimal time-window for the positive effects of estrogen to be observed that is related to lifetime estrogen exposure, ET initiation, and duration. In order to better characterize the punctual and long-term effects of estrogen, we included postmenopausal women currently using ET, past HT users, never users, and men.

2. Materials and methods

2.1. Participants

In total, 56 individuals (aged 50–74 years) answered ads in local newspapers to participate in the study approved by the Montreal Neurological Institute Ethic Committee and all provided written consent. Given the possible confounds linked to the healthy user's bias [23], [32] and [40], we obtained information about education level, incidence of current or recent depressive episodes, body mass index, exercise levels, alcohol intake, smoking habits, and income levels. Participants reported on
how they perceived their health, diet, and memory, and the number of doctor's visits for the past year. Participants also described the frequency of engaging in activities such as reading, watching TV, hobbies, walking, sports, exercise, napping, shopping, cultural and social activities, writing, etc. This was accomplished by screening all participants via telephone interviews and questionnaires.

Individuals who reported a history of cardiovascular, neurological or psychiatric conditions, diabetes, asthma, respiratory disease, thyroid dysfunction, adrenal dysfunction, arthritis, head trauma, and drug or alcohol problems were excluded from the study. Smokers were included only if they smoked less than 12 cigarettes per day. Participants were excluded from the study if they scored higher than 20 on the Geriatric Depression Scale (GDS) [39].

A total of 41 postmenopausal women were recruited. Of those, 16 were unopposed estrogen users, 10 were past-users, and 15 had never used HT. In addition, we recruited 15 men. Although the past-users group was significantly older than the three other groups, our participants were equivalent for education levels and GDS scores (see Table 1). The ET users were taking either conjugated equine estrogens (CEE) or estradiol therapy (oral or transdermal) and had been taking ET from 6 months to 27 years (see Table 2).
Table 1
The mean and standard deviation of demographic information and variables known to alter estrogen levels throughout woman’s life for ET users, past users, never users and men

<table>
<thead>
<tr>
<th></th>
<th>ET users n = 16</th>
<th>Past users n = 10</th>
<th>Never users n = 15</th>
<th>Men n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>58.9 ± 6.4</td>
<td>65.9 ± 5.5 *</td>
<td>59.9 ± 7.6</td>
<td>55.7 ± 4.0</td>
</tr>
<tr>
<td>Education</td>
<td>13.6 ± 2.5</td>
<td>12.9 ± 2.4</td>
<td>14.4 ± 3.4</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 4.1</td>
<td>24.3 ± 2.7</td>
<td>25.6 ± 5.3</td>
<td>26.3 ± 3.9</td>
</tr>
<tr>
<td>Geriatric Depression Scale (GDS)</td>
<td>3.5 ± 4.4</td>
<td>2.3 ± 1.8</td>
<td>4.9 ± 4.6</td>
<td>3.9 ± 4.5</td>
</tr>
<tr>
<td>Age at menses</td>
<td>12.8 ± 1.2</td>
<td>12.9 ± 1.8</td>
<td>13.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>1.9 ± 1.3</td>
<td>1.9 ± 1.4</td>
<td>2.2 ± 1.1</td>
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</tr>
<tr>
<td>Age at menopause</td>
<td>45.7 ± 7.1</td>
<td>48.6 ± 8.2</td>
<td>49.6 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Surgical menopause</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HT duration (years)</td>
<td>10.5 ± 9.3</td>
<td>8.2 ± 6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delay between menopause and HT</td>
<td>2.8 ± 5.4</td>
<td>0.4 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time since E exposure (years)</td>
<td></td>
<td>8.7 ± 8.3 (time since HT cessation)</td>
<td>10.3 ± 7.8 (time since menopause)</td>
<td></td>
</tr>
</tbody>
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Table 2

Descriptive variables of the ET users

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age last Menses (N or S)</th>
<th>Age start ET</th>
<th>Lag between M and ET (years)</th>
<th>Type of ET 1</th>
<th>Years on ET 1</th>
<th>Type of ET 2</th>
<th>Years on ET 2</th>
<th>Total years on ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET01</td>
<td>49 (S)</td>
<td>49</td>
<td>0</td>
<td>Estrace® 1 mg</td>
<td>1</td>
<td>Vivelle® 1 mg</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ET02</td>
<td>39 (S)</td>
<td>53</td>
<td>14</td>
<td>Premarin® 0.625 mg &amp; Provera®</td>
<td>9</td>
<td>Premarin® 0.625 mg</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>ET03</td>
<td>53</td>
<td>53</td>
<td>0</td>
<td>Estrace® 0.5 mg</td>
<td>1</td>
<td>Estrace® 1 mg</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ET04</td>
<td>50 (S)</td>
<td>50</td>
<td>0</td>
<td>Premarin® 0.625 mg</td>
<td>6</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>ET06</td>
<td>33 (S)</td>
<td>33</td>
<td>0</td>
<td>Premarin® 0.625 mg</td>
<td>8</td>
<td>Premarin® 1,25 mg</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>ET07</td>
<td>42</td>
<td>42</td>
<td>0</td>
<td>Premarin® 0.3 mg</td>
<td>4</td>
<td>Premarin® 0.625 mg</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>ET08</td>
<td>50 (S)</td>
<td>52</td>
<td>2</td>
<td>Estrogel® 2500 mg</td>
<td>2</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>ET10</td>
<td>32 (S)</td>
<td>50</td>
<td>18</td>
<td>Premarin®</td>
<td>3</td>
<td></td>
<td></td>
<td>3</td>
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<tr>
<td>ET12</td>
<td>52</td>
<td>52</td>
<td>0</td>
<td>Estring®</td>
<td>7</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>ET13</td>
<td>53</td>
<td>56</td>
<td>3</td>
<td>Premarin® 0.625 mg</td>
<td>0.5</td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>ET14</td>
<td>52</td>
<td>52.5</td>
<td>0.5</td>
<td>Premarin® 0.625 mg</td>
<td>6.5</td>
<td></td>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>ET15</td>
<td>48</td>
<td>53</td>
<td>5</td>
<td>Premarin® 0.325 mg</td>
<td>0.5</td>
<td>Estraderm® 0.025 mg + 0.05 mg</td>
<td>(3 + 3)</td>
<td>6</td>
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<tr>
<td>ET17</td>
<td>51</td>
<td>54</td>
<td>3</td>
<td>Premarin® 0.625 mg</td>
<td>0.5</td>
<td>Estraderm® 0.025 mg</td>
<td>14</td>
<td>14.5</td>
</tr>
<tr>
<td>ET18</td>
<td>38 (S)</td>
<td>38</td>
<td>0</td>
<td>Premarin® 0.625 mg</td>
<td>26</td>
<td></td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>ET19</td>
<td>41 (S)</td>
<td>41</td>
<td>0</td>
<td>Premarin® 0.625 mg</td>
<td>17</td>
<td>Premarin® 0.3 mg</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>ET20</td>
<td>48</td>
<td>48</td>
<td>0</td>
<td>Premarin®</td>
<td>21</td>
<td>Estrace®</td>
<td>5</td>
<td>26</td>
</tr>
</tbody>
</table>
2.2. Magnetic resonance image acquisition and preprocessing

MRI scans were obtained using a 1.5 T Siemens Magnetom Vision scanner with a standard radio frequency head coil. The volumes were acquired using a spoiled gradient echo sequence with TR of 22 ms; TE of 10 ms; flip angle of 30, and FOV of 224 mm x 256 mm x 160 mm. This protocol acquires T1 weighted images with a 1 mm isotropic resolution.

After acquisition, the native MR images were processed by a combination of algorithms for manual segmentation. The images were first corrected for intensity non-uniformity [34] and then registered into Talairach space [6]. This latter step accounts for individual differences in head size.

2.3. Manual segmentation of HC volume

The anatomical boundaries used for both HC and AG have been described in detail elsewhere [28]. In short, the following procedures for delineation of HC and AG were employed:

The most posterior part of the HC was defined as the first appearance of ovoid mass of gray matter inferiomedial to the trigone of the lateral ventricle (TLV). The lateral border of the HC at this point was the TLV, whereas medially, the border of the HC was identified by white matter. Further anterior, an arbitrary border was defined for the superior and medial border of the HC, in order to differentiate HC gray matter from the gray matter of the Andreas Retzius gyrus, the fasciolar gyrus, and the crus of the fornix.

For the HC body, the most visible inferirolateral layer of gray matter was excluded, assuming that it actually represents entorhinal cortex. Next, the white matter band at the superomedial level of the HC body, the fimbria, was included. If gray matter was found superior to the fimbria, the first row of gray matter was also included. The dentate gyrus, located in between the four CA regions in the hippocampal formation, together with the CA regions themselves and part of the subiculum, were included. The subiculum was divided by drawing a straight line with an angle of approximately 45° from the most inferior part of the HC medially to the
cistern if no white matter delineation was visible between these two structures. The lateral border at this point was identified by the inferior horn of the lateral ventricle.

The appearance of the HC head was defined by the emergence of the uncal recess of the HC head in the superomedial region of the HC. The most important structures for identification of lateral, anterior, and superior borders of the HC head were the uncal recess of the inferior horn of the lateral ventricle and the alveus. Besides the coronal view, the sagittal and horizontal views were employed for identification of the anterior border of the HC.

2.4. Manual segmentation of AG volume

The AG is located in the superomedial temporal lobe with parts of the basal ganglia from superior and entorhinal cortex from inferior blending in. The posterior end of the AG was defined in the coronal and horizontal planes, at the point where gray matter first started to appear superior to the alveus and laterally to the HC. If the alveus was not visible, the inferior horn of the lateral ventricle was employed as border. The superior border of the AG was arbitrarily defined by drawing a horizontal line between the superolateral part of the optical tract and the fundus of the inferior portion of the circular sulcus of the insula. This border was chosen to prevent erroneous inclusion of parts of putamen and claustrum in the amygdaloid measurement. For identification of the medial and lateral border, the horizontal view was employed. The ambient cistern was used as medial border, after excluding one layer of gray matter directly adjacent to the cistern, assuming that a clear separation from the cisternal area is impossible. Further anterior, a semicircle drawn from the lateral end of the lateral ventricle to the alveus was employed as an arbitrary landmark for the medial border, since the transition of AG to entorhinal cortex is not visible in MR images. The lateral border of the AG was defined by the lateral half of the semicircle. For the inferior border of the AG, the coronal images were employed for best separation. The tentorial indentation served as demarcation line between AG and entorhinal cortex, by excluding the gray matter inferolateral to the indentation. The anterior border of the AG was defined at the level of the closure of the lateral sulcus, which was identified in the horizontal plane.
2.5. Statistical analysis

We analyzed the data using a two factor (group by hemisphere) mixed design analysis of covariance with group (ET users, past users, never users, and men) as the between subject factor and with the volume of the specific structure in each hemisphere as the repeated measures dependent variable (HC&AG). Possible confounding variables were entered as covariates in subsequent analyses. Since the past users group was significantly older than the other groups, age was treated as a covariate in each analyses. The possible differential association between age and HC and AG volumes in the four groups was investigated using Pearson correlations. Finally, we performed correlational analyses between ET/HT duration and HC–AG volumes in the ET users and past users groups. All correlational analyses were corrected for multiple comparisons using the Bonferroni method. Finally, we performed exploratory analyses, using Mann–Whitney U-tests, to see if there was a differential effect of estrogen preparations (CEE versus estradiol) and type of menopause (natural versus surgically induced) within the ET users group on HC and AG volumes.

3. Results

3.1. HC and AG volumes

Analysis of variance revealed a significant interaction between group and HC side ($F (3,51) = 3.2, p < 0.03$). Post hoc analyses showed that ET users had a larger left HC volume compared to men, and a larger right HC volume compared to past users, never users, and men (all $p < 0.01$; see Fig. 1). The difference ranged from 8 to 11% in HC volume. There were no significant differences in AG volume between the groups ($p > 0.1$).
Hippocampal volume as a function of group

Figure 1. Hippocampal volumes of ET users ($n = 16$), past users ($n = 10$), never users ($n = 15$), and men ($n = 15$). All differences are significant at a level of $p < .05$, corrected for age. HC volumes are shown in mm$^3$.

3.2. Differential effects of age and variables altering E exposure and the window of opportunity

Correlational analysis revealed that the group of women currently using ET demonstrated a trend for an age-related decline in HC volume ($r_{\text{right hc}} = -0.47, p = 0.07$; $r_{\text{left hc}} = -0.42, p = 0.10$) whereas past users and never users did not show a significant HC volume change over time. In the male participants, a negative correlation between HC volume and age ($r_{\text{right hc}} = -0.47, p = 0.07$; $r_{\text{left hc}} = -0.56, p = 0.03$) was observed, similar to what we have previously demonstrated (Pruessner et al. [21]). However, after applying the Bonferroni correction none of these correlations remained significant. The amygdala volumes were not correlated with age in either women or men.

Interestingly, in the ET users group, a negative correlation between ET duration and HC volumes appeared ($r_{\text{HC right}} = -0.63, p < 0.01$ and $r_{\text{HC left}} = -0.52, p < 0.04$) showing that longer ET duration is associated with smaller HC volume. ET duration accounted for 40 and 27%, respectively, of the HC volume variance in the ET group. Fig. 2 shows the relation between ET duration and mean (left and right) hippocampal volume in the group of women using ET. Importantly, HT duration was not related to HC volume in the past-users group. The AG volume did not correlate with treatment duration. We also performed correlation between age at menses, age
at menopause and HC–AG volumes but no significant relation were found (all \( p > 0.20 \)).

Figure 2. Scatterplot showing the negative association between total HC volumes and ET duration in the group of 16 ET users. ET duration ranged from 6 months to 27 years. HC volumes are shown in mm\(^3\).

Finally, our data did not allow exploring the effect of time of initiation of HT. The delay between menopause and onset of treatment was non-significant between the current and past users. Further, the majority of our subjects had no delay between menopause and treatment; the number of subjects who delayed onset of HT after menopause was too small and scattered to allow investigation of this question (see Table 1 and Table 2, delay between menopause and HT/ET).

### 3.3. Exploratory analysis of ET preparations and type of menopause

In addition, the ET users group was split into CEE users (\( n = 9 \)) and estradiol users (\( n = 7 \)). Using Mann–Whitney \( U \)-tests on the HC and AG volumes, we failed to observe significant differences between CEE users and estradiol users in the ET users group (all \( p > 0.20 \)).

Grouping women based on their type of menopause (surgically or naturally induced) and comparing their HC and AG volumes using the Mann–Whitney procedure, no significant group differences was observed, neither when all women
were included in the analyses nor when only the ET users were analyzed (all \( p > 0.20 \)).

3.4. Confounding variables

To explore the potential impact of confounding variables on the group composition, we employed a series of one-factor (group) analyses of variance. There was no significant difference between the groups (all \( p > 0.2 \)) for education levels, body mass index (BMI), Geriatric Depression Scale scores (GDS), alcohol consumption, income levels, and number of doctor appointments per year. Also, participants did not differ on how they perceived their health, diet and memory (all \( p > 0.05 \)). In addition, there was no significant group difference (all \( p > 0.05 \)) on any questions related to their activities including the frequency of walking, exercise, sports, social activities, and overall mean activities.

4. Discussion

In this study, we investigated the possible protective effects of ET on brain structures known to contain a high-density of classical nuclear ER and to be involved in memory function, namely the HC and the AG. Findings from a number of studies investigating the effects of HT on memory following menopause have noted that estrogens could exert neuroprotective effects in the aging process [40]. In line with these findings, we found larger right HC volumes in ET users compared to past-users, never-users, and men, and larger left HC volume in ET users compared to men. This finding is consistent with the notion that using ET following menopause is beneficial for women with regards to the integrity of specific brain structures related to memory, and potentially memory function as well. These findings complement the preliminary findings by Eberling et al. [8].

Recent neuroimaging studies have reported both gender and age-related differences in a variety of brain regions [5], [7], [14], [16], [17], [18], [26] and [38]. Our previous results (Pruessner et al. [26]) showed that in men, the volume of the HC declines linearly after the third decade of life, whereas HC volume remains stable in women of this age. It is thus possible that in women, endogenous estrogen exposure
prior to menopause may contribute to the preserved HC volume. This is consistent with the notion of estrogen-conferred neuroprotection.

Yet, in the current study, although women using ET had larger overall HC volumes, they showed a trend for a linear decline in HC volume with age in comparison to past users and never users who demonstrated a constant HC volume over time. Interestingly, the HC volume decline in the ET group was even more pronounced when plotting it against ET duration thus raising the possibility of a treatment duration effect. Here, it could be speculated whether the use of ET following menopause might only be beneficial for a certain period of time. Evidence from in vitro and in vivo studies allows us to speculate about the possible mechanisms of action of estrogen on the brain. The results of our study suggest that the protective effect of estrogens on cerebral structures might not be mediated solely by the relative expression level of nuclear ER because of the observed differential effect of ET in the HC and the AG since both structures are characterized by high nuclear ER density.

Recently, some data suggested that other ER exist such as the membrane-associated estrogen receptors. Even though they are currently not well understood, functional evidence suggests their importance in neural target cells of estrogen [37]. Evidence also exists suggesting that estrogen might act as a chemical shield by protecting neurons against a wide range of acute and age-related insults such as amyloid-β toxicity and glutamate excitotoxicity in the hippocampus particularly in a dose-dependent manner [2], [3] and [24].

In vivo evidence showed that estrogen enhances synaptic plasticity, dendritic spine density and connectivity in the HC [2], [24], [35] and [36]. Of importance also is the neurotrophic effects of estrogens [37]. Estrogens have profound influence on neural survival of other growth factors such as brain-derived neurotrophic factors (BDNF) [37] and promote neurogenesis in the dentate gyrus of the HC [15] and [36]. Combined, these factors could explain the overall higher HC volume levels in the ET user group, but cannot account for the observed decline of HC volumes with ET duration.
One possible explanation is that estrogens may have time-dependent effects, given that prolonged absence of hormones and chronic activity of the nuclear ER have both been showed to lead to decline in functionality [12]. Another possible explanation is the differential consequences of constant versus fluctuating estrogen levels on the brain. Continuous administration of HT following menopause could possibly contribute through interaction with the immune system to exacerbate normal dysfunctions associated with aging or trigger pathological aging [22]. Taken together, in vitro and in vivo results raise several potential mechanisms by which estrogens could have temporary neuroprotective function on the brain particularly on hippocampal cells, thus providing some molecular explanations regarding the possible transient beneficial effect observed on HC volumes in the ET users group.

The inclusion of a group of past users in the current study provides additional information about the potential long-term protective effect of estrogen on the brain after treatment cessation. As a group, the past users did not differ significantly from the never users in HC volumes. This finding suggests that there is no potential long-term effect of HT after cessation of the therapy on HC volumes. However, the scattered distribution of the HC volumes among this group, as seen by the larger standard deviations and on the graphical representation (see Fig. 1), call into question the potential long term beneficial effect of estrogens after treatment cessation previously observed by other [4] and [9]. Due to the small sample size of this group in particular (n = 10), further investigations are required to better understand the possible long-term effect of ET after treatment cessation.

The overall larger HC volumes observed among the ET users raise the question whether ET might prevent cognitive decline in late adulthood. Having larger HC volumes might not be functionally significant early in the aging process but might be beneficial in late adulthood. The fact that HC volume decline is a known risk factor for the development of mild cognitive impairment (MCI), and Alzheimer's disease (AD) would support this view [1] and [19]. If the current observation of a duration effect can be supported in future studies, the question then would be if the delay in HC volume decline brought upon by ET is also effective in delaying the onset of MCI and AD for these populations. Again, this would be for future studies to investigate.
Factors such as the onset of hormone therapy, previous exposure to endogenous or exogenous estrogen, and duration of treatment might be of importance in order to better understand the influence of estrogens on the brain and cognition. The small sample size and the cross-sectional nature of the current study did not allow us to address all of these questions, thus future studies would have to look at this more closely as well. We also explored whether variables known to alter lifetime estrogen exposure, such as age at menses and age at menopause, would have an effect on HC volumes, but no relation was observed with any of the investigated brain structures in our sample.

There are a number of limitations in the current study. Firstly, the study design suffered from a small sample size within the different groups of users and non-users, and men. However, our results replicate results of others with regard to effects on the HC [8], and the observed effects seem to be relatively robust. Secondly, our analysis of brain structures concentrated on the HC and AG volumes. Future studies would certainly benefit from either manual segmentation protocols of other brain areas (e.g., frontal cortex or entorhinal and perirhinal cortices [27], or cortical thickness [20]). Thirdly, even if we demonstrated in this study that women using ET were not different on variables linked to a potential health bias effect compared to other postmenopausal women, there are other variables such as current fitness levels [9], personality factors (e.g., self-esteem) [25] or the presence of menopausal symptoms that could explain volume differences, or differences in treatment-seeking behavior. However, these variables were not assessed systematically in the current study, and thus we cannot exclude the possibility that these factors might explain a systematic proportion of the variance. Future studies should try and include a more thorough assessment of these variables as well. It is conceivable that ET users might still represent a particular group of women with specific demographic or personality differences, and that this could account for part of the observed brain volume differences.

In summary, the current findings support a neuroprotective role of estrogens on hippocampal volume in aging. However, the finding of a strong negative correlation between ET duration suggests that this neuroprotection might only be temporary, and might loose its effectiveness after a specific period of time. Finally,
the observed effects appear to be region-specific for the HC given that no significant differences were found in the AG.

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Chapter 4:
Endogenous and Exogenous Estrogen Exposure Affect
Brain Regions Involved in Sexual Behavior and Memory:
a Voxel Based Morphometry study

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& Jens C Pruessner, Ph.D.
Preface to chapter 4

The last manuscript had for main goal to assess if the effects of estrogens on the brain are specific to the hippocampus since we observed a region-specific effect of ET use on this cerebral structure in the previously described study and if they can be linked to endogenous and exogenous estrogen exposure indices.

In the current study, we assessed the specificity of this ET effect by investigating the whole brain using voxel-based morphometry (VBM). Voxel-based morphometry is an appropriate technique to explore these effects since it allows us to investigate the morphology of the whole brain in a fairly short amount of time. In our case, it also provided us the unique opportunity to see if we could confirm the HC volume findings and assess every regions that contains estrogen receptors even the one that are difficult to assess using more precise techniques such as manual segmentation.

The fifty-six previously investigated subjects were entered in serial optimized VBM analyses using group and sex as independent variables, and age and variables of lifelong estrogen exposure as covariates. As expected we found that, compared to men, ET users have greater grey matter (GM) concentration in the right HC and that ET duration is negatively correlated with GM density in the right HC. Interestingly, we specify the group effect to the anterior part of the HC and the duration effect to the posterior part of the HC. We further show that ET users compared to never users have greater GM concentration in the left nucleus accumbens (NAcc) and that this effect is modulated by age at menarche and menopause. Investigating sex differences, we find that women have larger right NAcc, hippocampi, inferior temporal gyri and prefrontal cortices compared to men. These results point towards the existence of neuroprotective effects of both endogenous and exogenous estrogens on brain regions outside the hippocampus.
Manuscript

Endogenous and Exogenous Estrogen Exposure Affect Brain Regions Involved in Sexual Behavior and Memory: a Voxel Based Morphometry study.

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Estrogen, hormone therapy (HT), brain, aging, Magnetic Resonance Imaging (MRI), Voxel-based Morphometry (VBM)
Abstract

It is still a matter of debate whether estrogen can have a protective effect on brain integrity. We previously demonstrated that postmenopausal women currently using estrogen therapy (ET) had larger hippocampal (HC) volumes compared to past users, never users, and men. We further demonstrated that ET duration was negatively associated with HC volumes. In the current study, we assessed the specificity of this ET effect by investigating the whole brain using voxel-based morphometry (VBM). High-resolution structural Magnetic Resonance Imaging (MRI) scans of 56 previously investigated subjects were entered in serial optimized VBM analyses using group and sex as independent variables, and age as covariate. We can demonstrate that, compared to men, ET users have greater grey matter (GM) concentration in the right anterior hippocampus and that ET duration is negatively correlated with GM density in the right posterior hippocampus. We further show that ET users compared to never users have greater GM concentration in the left nucleus accumbens (NAcc) and that this effect is modulated by age at menarche and menopause. Investigating sex differences, we find that women have larger right NAcc, hippocampi, inferior temporal gyri and prefrontal cortices compared to men. These results point towards the existence of neuroprotective effects of both endogenous and exogenous estrogens on brain regions outside of the hippocampus.
Introduction

Hormonally-based treatments have existed for over a century to alleviate physiological symptoms linked to the menopausal transition. The debate whether the effects of post-menopausal hormone therapy (HT) on health are indeed beneficial has been going on for over 40 years (Stefanick 2005) and has recently been rekindled by the results of the Women Health Initiative (WHI) study. The conclusions from the WHI studies, which argue that there would be more health risks than benefits in using HT, unsettled the scientific community and particularly the neuroscientists interested in the effects of estrogens and post-menopausal hormonal treatment. Classically, post-menopausal hormone therapy (HT) consists of either unopposed estrogens (ET) or a combination of estrogens and progestogens (EPT). Estrogens are well known for their lifelong role in development, maintenance of primary sexual physiology and reproductive as well as sexual behaviours but also for their potential neuroprotective effects on brain integrity and cognitive functions. The last few years provided new data on the effects of estrogens on the brain and cognition in aging, particularly with the advent of neuroimaging techniques.

It has been repeatedly observed that the velocity of age-related gray matter loss is greater in men than in women (Cowell, Turetsky et al. 1994; Coffey, Lucke et al. 1998; Gur, Turetsky et al. 1999; Xu, Kobayashi et al. 2000; Goldstein, Seidman et al. 2001; Pruessner, Collins et al. 2001; Raz, Gunning-Dixon et al. 2004), with some studies reporting inconclusive results (Resnick, Pham et al. 2003; Lemaitre, Crivello et al. 2005; Smith, Chebrolu et al. 2006). Interestingly, the studies reporting no difference often assessed older individuals (mean age of 70 and more), questioning the linearity of the relationship between age and sex-related brain volume difference. In addition, the cross-sectional nature of most of these studies could add cohort effects which are difficult to control for. Moreover, the relevant studies are difficult to compare because of different age-ranges, different inclusion criteria, different brain regions of interest, and different analyses techniques. Therefore, there is no consensus among studies which regions are most affected by estrogens or sex in aging.

One possible explanation that has repeatedly been put forward in order to explain the sexual dimorphism in the pace of brain aging is the implication of gonadal steroids such as estrogens. Based on animal research, it has recently been
hypothesized that it might be linked to the known sex difference in estrogen exposure during brain development (Goldstein, Seidman et al. 2001). It has also been attributed to lifelong or current differences in circulating estrogen levels with women having higher estrogen levels and exposure than men prior menopause (Cowell, Turetsky et al. 1994; Coffey, Lucke et al. 1998; Gur, Turetsky et al. 1999; Xu, Kobayashi et al. 2000; Pruessner, Collins et al. 2001; Raz, Gunning-Dixon et al. 2004).

Accordingly, studies emerged on the link between hormone therapy and gray matter integrity in aging. To this date, less than a dozen studies assessed the possible neuroprotective effects of HT in post-menopausal women, all of these studies using different techniques and region of interests (Eberling, Wu et al. 2003; Raz, Rodrigue et al. 2004; Erickson, Colcombe et al. 2005; Boccardi, Ghidoni et al. 2006; Ghidoni, Boccardi et al. 2006; Erickson, Colcombe et al. 2007). Overall, results point towards selective sparing of grey matter in postmenopausal women using HT. In a recent study, our group demonstrated that ET users had larger hippocampal (HC) volumes compared to past-users, never-users and men. On the other hand, we demonstrated that ET duration was negatively related to HC volume, given that women using ET for a longer period of time had smaller HC volumes. Interestingly, despite the fact that the amygdala is also a high density estrogen receptor region, we did not find significant volume difference in this structure (Lord, Buss et al. in press). Based on these findings, the goal of the present study was to assess the specificity of the estrogen effect on the aging brain in the same population by investigating the impact of sex and ET on gray matter density using Voxel-Based Morphometry (VBM). Variables such as age at first menstruation and age at menopause were included in the analysis to take into account a possible lifelong estrogen exposure effect. We hypothesized that postmenopausal women would exhibit more GM preservation in certain brain regions compared to men, that it would be more striking in the group of ET users and modulated by lifelong estrogen exposure markers.
Materials and Methods

Participants

We examined 56 individuals (aged 50-74 years) recruited via a newspaper ad placed in local journals. A total of 41 postmenopausal women were recruited. Of those, 16 were unopposed estrogen users (ET), 10 were past-users, and 15 had never used HT. In addition, we recruited 15 men. This is the same population that we recently investigated for differences in hippocampal volumes (Lord et al., in press). Although the past-users group was significantly older than the 3 other groups, our participants were equivalent regarding education level and Geriatric Depression Scale (GDS) score (Yesavage, Brink et al. 1982). The ET users were taking either conjugated equine estrogens (CEE) or estradiol therapy (oral or transdermal) and had been taking ET from 6 months to 27 years. (see Table 1).

Given the possible confounds, information about education level, incidence of current or recent depressive episode, body mass index, exercise level, alcohol intake, smoking habit, and income level was obtained. This was accomplished by screening all participants via telephone interviews and questionnaires. However, we did not observe group difference on any of the confounding variables measured. Individuals who reported a history of cardiovascular, neurological or psychiatric conditions, diabetes, asthma, respiratory disease, thyroid dysfunction, adrenal dysfunction, arthritis, head trauma, and drug or alcohol abuse were excluded from the study. Smokers were included only if they smoked less than 12 cigarettes per day. Participants were excluded from the study if they scored higher than 20 on the GDS. This study was approved by the Montreal Neurological Institute (MNI) ethic committee and all participants provided informed written consent.

MRI acquisition and Analysis

MRI scans were obtained using a 1.5 T Siemens Magnetom Vision scanner with a standard radio frequency head coil. The volumes were acquired using a three-dimensional spoiled gradient echo sequence with sagittal volume excitation (repetition time ($T_R$) of 22 ms; echo time ($T_E$) of 10 ms; flip angle of 30°) and a field of view (FOV) of 224x256x160 mm. This protocol acquires $T_1$-weighted images with a 1 mm isotropic resolution.
To prepare the original images for the optimized VBM analysis, the native MR images were preprocessed with combination of algorithms using software developed at the MNI. The images were first corrected for intensity non-uniformity (Sled, Zijdenbos et al. 1998) and registered into standard stereotaxic space (Collins, Neelin et al. 1994). This latter step aligns the images along the AC-PC axis and accounts for individual differences in brain size and shape. Next, the images were classified into gray matter, white matter and cerebrospinal fluid (CSF) using an automated tissue classifier (Zijdenbos, Forghani et al. 1998). Further analyses were conducted on the extracted gray matter maps. The cerebellum, skull, and dura were masked for sake of clarity and to avoid misclassification of nonbrain voxels. These images were then blurred using a Gaussian smoothing kernel (full width at half maximum (FWHM), 10mm) to increase the signal-to-noise ratio, accommodate for individual differences and reduce the number of comparisons for the regresional analysis. VBM analysis was conducted using the glim-image software package developed at the MNI. An average brain of the corrected files was created in order to visualise the results. In order to optimize the analysis, another average brain was created with the blurred files and use as a mask on the result files to include only voxels which have intensity values above 0.1.

Statistical analysis

Analyses were processed using the smoothed gray matter maps to identify regional morphological differences. The subject’s group/sex/ET duration acted as independent variables and the MR image signal intensity of each voxel acted as a dependent variable. Since we wanted to observe the specific effects of ET and sex on the brain, and because the past users group was significantly older than the other groups, age was treated as a covariate. A t-statistic map was obtained based on the slope of the regression of the gray matter density at each voxel against the grouping factors. This method, based upon Gaussian random field theory, corrects for multiple comparisons in a given search volume, in this case the gray matter maps of all subjects (Worsley, Marret et al. 1996). To reach a significance levels of p<0.05 corrected for multiple comparisons, t-values were threshold at 3.4, given a df of 100 and 10mm of FWHM. In every analysis assessing only the women we also included age at menopause and age at first menstruation in order to account for possible
effects of lifelong endogenous estrogen exposure. The relation between ET duration in the ET users group and gray matter density was also assessed using regression analysis.

In addition, based on the classified images we extracted the whole gray and white matter volumes and performed analyses of covariance with group (4 groups, 3 groups of women or sex) as the between subject factor, age as the covariate, and either total white or gray matter volume as the dependent variable.

Results

VBM Analysis

ET users, past users, never users, and men

As hypothesized, whole-brain VBM analysis revealed that ET users show greater grey matter (GM) density in the right hippocampal head and entorhinal cortex compared to men (at the peak value, F(3, 54)= 4.1 p<0.01) (See Fig.1; Table 2). We also observed that the 3 groups of women (ET users, past and never users) have greater GM concentration in the right nucleus accumbens (NAcc) compared to the men (significance at peak, F(3, 54)= 3.9 p<0.01). (See Fig.2; Table 2)

ET duration

ET duration in the group of ET users was negatively associated with GM matter density in the right hippocampus tail (r = -0.48, p<0.05, correlation between GM matter density at the peak t-value and ET duration) and in the right posterior parahippocampal gyrus (r = -0.66, p<0.01) (See Fig.3; Table 2).

ET users, past users and never users

Assessing women only, we found that ET users have greater GM density in the left NAcc compared to the never users and that this effect was modulated by age at first menses (fm) and age at menopause (lm). (3 groups, age, fm & lm at the peak value, F(2, 40)= 2.4 p<0.05). (See Fig.4; Table 2)
Sex difference (Women without ET users vs men)

We observed significant sex differences with women having greater GM concentration in the right NAcc and bilateral HC, inferior temporal gyri and medial prefrontal cortex (at peak levels, $F_{\text{NAcc}}(1, 38) 7.7, p<0.01$; $F_{\text{HC}}(1, 38) 8.4, p<0.01$; $F_{\text{HC}}(1, 38) 3.9, p<0.05$; $F_{\text{ITG}}(1, 38) 7.0, p<0.01$; $F_{\text{ITG}}(1, 38) 4.7, p<0.05$; $F_{\text{PFC}}(1, 38) 5.7, p<0.01$; $F_{\text{PFC}}(1, 38) 5.6 p<0.01$).(See Fig. 5; Table 2)

Whole WM-GM volume

We did not observe a significant difference on global tissue volumes for both the gray matter and the white matter neither between women and men nor between users and non-users (all $p>0.05$). Also, there was no significant correlation between ET duration and both total GM and WM volume in the ET users group ($p>0.05$).

Discussion

The delineated results confirm and extend previous observations from our group and demonstrate a unique pattern of disparity in GM density among postmenopausal women and between sexes. We replicated and specified previously reported findings (Eberling, Wu et al. 2003; Lord, Buss et al. in press) by demonstrating higher GM concentration in the right hippocampal head and entorhinal cortex of ET users compared to men. In Lord et al. (in press) we demonstrated that ET users had larger right HC volume compared to past users, never users, and men but did not assess which part of the HC was more preserved. We were also able to specify the previously observed negative correlation between HC volume and ET duration among ET users to the most posterior part of the HC. So far, these results demonstrate a selective sparing of GM concentration inside the HC itself, the anterior part showing robust neuroprotective effects of estrogen, whereas the tail seems to show signs of aging or estrogen assault after certain treatment duration.

This argues in favour of both the anterior and the right-greater-than-left location of HC atrophy in AD previously described (Jack, Petersen et al. 1997). Interestingly, ET use has been repeatedly reported to decrease the risk or delay the onset of AD but no consensus exists on the possible underlying mechanisms (Henderson 1997; Fillit 2002). The observed greater GM density in the right HC head and entorhinal cortex in ET users might provide support for the latter idea of estrogen
neuroprotection on memory function and against early AD assaults following menopause. On the other hand, the constant administration of ET might lead to a decrease in receptor sensitivity, which in turn could explain the transient beneficial effect of estrogen on the HC. Studies suggest that extended exogenous estrogen exposure might cause a decrease in its known genomic neuroprotective functions against aging by decreasing estrogen receptors efficacy (Marriott and Wenk 2004; Foster 2005), exacerbating normal dysfunctions associated with aging or triggering pathological aging through immune system interactions (Marriott and Wenk 2004).

Besides enhancing neurons survival and modulating cognitive function, estrogens modulate the actions of several neurotransmitters such as acetylcholine, serotonin, noradrenalin and dopamine. These neurotransmitters along with estrogens are involved in a multitude of functions including thermoregulation, mood adjustment, sexual behaviour, and more. In the current study, we found an effect of both endogenous and exogenous estrogen exposure on GM density in the nucleus accumbens (NAcc). The NAcc is best known for its role in dopamine regulation in the context of reward and addiction (Ikemoto and Panksepp 1999). Our data suggest a relationship between estrogen exposure and GM density: Women with ET exhibited significantly higher left NAcc GM density than never users (exogenous estrogen effect), and women generally exhibited significantly higher right NAcc GM density than men (endogenous estrogen effect). Interestingly, the exogenous estrogen effect was also modulated by endogenous estrogen such that the influence of ET was stronger when markers of endogenous estrogen exposure (namely age at menarche and age at menopause) were taken into account. To the best of our knowledge, this is the first study to show an association between lifelong estrogen exposure and NAcc structure in humans.

The NAcc is a projection area of mesolimbic dopamine neurons originating in the ventral tegmental area along with the hippocampus and amygdala. NAcc dopamine activity is thought to play a pivotal role in the mediation of motivated behaviour and the reinforcing effects of rewarding stimuli (for a review see (Ikemoto and Panksepp 1999; Schultz 2002). At this point, we can only speculate on the mechanisms by which increased estrogen exposure throughout lifetime may act on NAcc GM density. Given the strong dopaminergic innervations of the NAcc, the known age-related decrease in dopamine function and regulation (Backman, Nyberg
et al. 2006), evidence from animal studies showing sex differences in striatal dopamine activity, variation associated with estrous cycle and effect of exogenous administration of estrogen (Thompson and Moss 1994; Becker 1999; Thompson, Bridges et al. 2001) it may be suggested that dopamine is implicated in the association between estrogen exposure and NAcc GM density. Interestingly, in female rats, it has been hypothesized that the hormonal modulation of striatal regions (including the NAcc) is evolutionary important since it may serve to facilitate reproductive success by enhancing pacing behaviour (Becker, 1999). In postmenopausal women, however, reproductive success is not an issue anymore since it is the end of a woman's reproductive life. Interestingly, following menopause sexual difficulties are among the most frequent reported health problems linked to menopause. It is reported that postmenopausal women experience decrease in sexual desire, arousal, orgasm, and frequency in sexual activities (Dennerstein, Dudley et al. 2001) linked to estrogens depletion associated with menopause (Dennerstein, Randolph et al. 2002). The use of ET efficiently restores sexual appetite, frequency and pleasure (Alexander, Kotz et al. 2004). With this in mind it would be interesting to test if in post-menopausal women the restoration of sexual appetite brought on by ET is mediated by the dopamine-enhancing effect of estrogen in the NAcc.

Unfortunately, at this point we were unable to find a satisfactory explanation for the observed asymmetrical findings in the GM matter concentration of the NAcc related to endogenous and exogenous estrogen exposure. Future studies will need to investigate potential functional implications of this hemispheric asymmetry.

Apart from suffering from a relatively small sample size, this study does not allow us to drive clear conclusions on the functional implications of the observed neuroprotective effect of estrogen on the HC and NAcc since behavioural measures are not available. Future studies should investigate treatment seeking behaviours as well as menopausal symptoms along with cognitive function more thoroughly. Moreover, even though in this study, we demonstrated that women using ET were not different on variables such as education, exercise level and income, other variables such as current fitness level (Erickson, Colcombe et al. 2007) and personality factors exist (e.g., self-esteem) (Pruessner, Baldwin et al. 2005) that could partly explain the observed differences.
Finally, even though VBM was used in its optimized form in the current study, results need to be interpreted with caution due to limitations inherent to the VBM technique. VBM has the advantage of being a fully automated, unbiased, and timesaving procedure. It is, however, unclear whether the locally observed differences are due to volumetric differences (size of the structure) per se, to a displacement of the structure between subjects/groups (location of the structure), or to the way the structure is formed between subjects/groups (shape of the structure). However, based on the goal of the current study, namely investigating the specificity of estrogens and uncover other possible regions apart from the HC that could be affected, VBM turned out to be a suitable method. It confirmed previous findings and identified the NAcc as a new but plausible region, which would have been difficult to assess using a manual segmentation protocol.

In sum, our results point toward a specific effect of estrogen on particular brain structures, the HC and the NAcc. We also demonstrate that lifelong estrogen exposure seems to differentially influence the effect of estrogens. Markers of endogenous estrogen exposure such as age at menarche and menopause as well as ET duration play a unique role in the understanding of the effect of estrogens on the brain and may help solve the current debate in estrogen research. Our results demonstrated that efforts should be put in order to better characterize the interaction between estrogens and the NAcc region and neurotransmitters in humans such as dopamine.
Acknowledgements
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Disclosure Statement
All the authors of this paper do not have any potential conflict of interest, being financial, personal or other. None of the authors’ institution has contracts relating to this research or has any other agreements that could be seen as involving a financial interest in this work. The study was performed after approval by the Montreal Neurological Institute (MNI) research ethics committee.
References


Table 1

The mean and standard deviation of demographic information and variables known to alter estrogen levels throughout woman’s life for ET users, past users, never users and men. The past users group was significantly older than the 3 other groups (*p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>ET users</th>
<th>Past users</th>
<th>Never users</th>
<th>Men</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 16</td>
<td>n = 10</td>
<td>n = 15</td>
<td>n = 15</td>
</tr>
<tr>
<td>Age</td>
<td>58.9 ± 6.4</td>
<td>65.9 ± 5.5 *</td>
<td>59.9 ± 7.6</td>
<td>55.7 ± 4.0</td>
</tr>
<tr>
<td>Education</td>
<td>13.6 ± 2.5</td>
<td>12.9 ± 2.4</td>
<td>14.4 ± 3.4</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 4.1</td>
<td>24.3 ± 2.7</td>
<td>25.6 ± 5.3</td>
<td>26.3 ± 3.9</td>
</tr>
<tr>
<td>Geriatric Depression Scale (GDS)</td>
<td>3.5 ± 4.4</td>
<td>2.3 ± 1.8</td>
<td>4.9 ± 4.6</td>
<td>3.9 ± 4.5</td>
</tr>
<tr>
<td>Age at first menses (fm)</td>
<td>12.8 ± 1.2</td>
<td>12.9 ± 1.8</td>
<td>13.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>1.9 ± 1.3</td>
<td>1.9 ± 1.4</td>
<td>2.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Age at menopause (lm)</td>
<td>45.7 ± 7.1</td>
<td>48.6 ± 8.2</td>
<td>49.6 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Surgical menopause</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HT duration (years)</td>
<td>10.5 ± 9.3</td>
<td>8.2 ± 6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delay between menopause and HT</td>
<td>2.8 ± 5.4</td>
<td>0.4 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time since E exposure (years)</td>
<td></td>
<td>8.7 ± 8.3 (time since HT cessation)</td>
<td>10.3 ± 7.8 (time since menopause)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2
Areas where there were significant differences in gray matter density. The $x$, $y$, $z$ are the coordinates in Talairach space of the location of the voxel with the highest significance (t-value at the peak).

<table>
<thead>
<tr>
<th>Groups/Condition</th>
<th>Cortical region</th>
<th>$x$</th>
<th>$y$</th>
<th>$z$</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 groups + age</td>
<td>Right Nucleus Accumbens</td>
<td>18</td>
<td>5</td>
<td>-13</td>
<td>-3.4</td>
</tr>
<tr>
<td>4 groups + age</td>
<td>Right hippocampus head</td>
<td>30</td>
<td>-10</td>
<td>-33</td>
<td>-3.6</td>
</tr>
<tr>
<td>3 groups of women + age + age at first menses (fm) + age at menopause (lm)</td>
<td>Left Nucleus Accumbens</td>
<td>-9</td>
<td>-2</td>
<td>-6</td>
<td>-3.7</td>
</tr>
<tr>
<td>ET duration in ET users group</td>
<td>Right HC tail</td>
<td>21</td>
<td>43</td>
<td>-2</td>
<td>-4.5</td>
</tr>
<tr>
<td>Sex (no ET users) + age</td>
<td>Right parahippocampal gyrus</td>
<td>32</td>
<td>42</td>
<td>-16</td>
<td>-4.6</td>
</tr>
<tr>
<td>Sex (no ET users) + age</td>
<td>Women &gt; Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Nucleus Accumbens</td>
<td></td>
<td>21</td>
<td>6</td>
<td>-17</td>
<td>-5.1</td>
</tr>
<tr>
<td>Right hippocampus head</td>
<td></td>
<td>23</td>
<td>-9</td>
<td>-26</td>
<td>-4.5</td>
</tr>
<tr>
<td>Left hippocampus</td>
<td></td>
<td>-26</td>
<td>-7</td>
<td>-28</td>
<td>-4.4</td>
</tr>
<tr>
<td>Right inferior temporal gyrus</td>
<td></td>
<td>45</td>
<td>10</td>
<td>-36</td>
<td>-4.2</td>
</tr>
<tr>
<td>Left inferior temporal gyrus</td>
<td></td>
<td>-45</td>
<td>-9</td>
<td>-33</td>
<td>-3.9</td>
</tr>
<tr>
<td>Right medial prefrontal gyrus</td>
<td></td>
<td>15</td>
<td>62</td>
<td>-13</td>
<td>-4.6</td>
</tr>
<tr>
<td>Left medial prefrontal gyrus</td>
<td></td>
<td>-7</td>
<td>62</td>
<td>-8</td>
<td>-4.1</td>
</tr>
</tbody>
</table>
Figure 1 ET users have greater grey matter (GM) density in the right hippocampus head and entorhinal cortex compared to men.

Figure 2 The 3 groups of women (ET users, past and never users) have greater GM concentration in the right nucleus accumbens (NAcc) compared to the men.
Figure 3 ET duration in the group of ET users was negatively associated with GM matter density in the right hippocampus tail and parahippocampal gyrus.

![Graph showing the association between ET duration and GM density in the right hippocampus tail and parahippocampal gyrus.](image)

$r = -0.48, p<0.05$

$r = -0.66, p<0.01$

Figure 4 ET users have greater GM concentration in the left NAcc compared to the never users, effect modulated by age at first menses (fm) and age at menopause (lm).

![Graph showing the GM concentration in the left NAcc for different groups of ET users.](image)
Figure 5 Significant sex differences in the NAcc, HC, inferior temporal gyri, and medial prefrontal cortex. (W>M)
Chapter 5:
Summary, general discussion and conclusion
General discussion and conclusion

Summary

Along the process of this thesis, our goal was to investigate the effect of estrogen on the aging brain in a novel and unique perspective. This led us to develop a questionnaire assessing the different markers of lifelong estrogen exposure in a woman’s life and include it in our studies, which was original and unique at the time. Serendipity came along and in May 2002, the largest randomized controlled trial study, the women Health Initiative (WHI) study, was abruptly interrupted since they found that HT was causing more harm than benefits on women’s health. Already five years since the first WHI publication, we can now say that the wind has changed and that the literature on the effect of HT on health as taken a turn in my line of thoughts.

In this thesis, I first presented in the introduction a review of the literature incorporating evidence from basic neuroscience, and from rat, nonhuman primate, and human studies that supports the theory that lifelong estrogen exposure is a crucial element when it comes to understanding the effect of estrogen on the brain and its functions. Secondly, the results of three studies were presented as independent articles; the first reporting the results of the reliability assessment of our in-house questionnaire «Indices of estrogen exposure» and the second and third presenting the results of two studies investigating the impact of current estrogen use and indices of lifelong estrogen exposure on the aging brain using two different techniques namely manual segmentation of the hippocampus and amygdala and a whole brain analysis technique, voxel-based morphometry.

The first article was the key in establishing if markers of lifelong estrogen exposure are reliable measures to use in order to test our hypotheses about the impact of lifelong estrogen exposure as a modulator of brain aging. A questionnaire was first developed to gain information about women lifelong exposure to estrogen assessing both endogenous and exogenous factors and was filled twice by postmenopausal women within a four year interval. Interestingly, we found that most lifelong estrogen exposure indices were reliable. An effect of age on the recall of these indices was also observed, with the older individuals being less or not reliable. However, age at menopause and age at hormone therapy initiation led to weaker correlations across time of measurements driven by the lack of reliability of the older
women. These results led us to the conclusion that most indices of estrogen exposure are reliable and gathering information from physician/pharmacy records when available could improve reliability when needed.

In the second article we investigated the potential neuroprotective effects of estrogens on brain regions with high levels of estrogen receptors, namely the hippocampus (HC) and the amygdala (AG) in postmenopausal women currently using unopposed ET, past HT users, never users, and men. Age at menses, age at menopause, HT duration and age were included as covariates in the analysis. We demonstrated that women using ET had larger left and right HC volumes compared to men, and larger right HC volumes compared to past users and never users. Moreover, we found a negative relationship between ET duration and HC volume in the ET user group. This allowed us to support the notion that there is a treatment duration-dependent neuroprotective role of estrogen on HC volume in aging pointing toward a window of opportunity.

The last manuscript of this thesis added credibility to the volumetry results by confirming, specifying and extending them. We found that compared to men, ET users have greater grey matter (GM) concentration in the right anterior HC and that ET duration is negatively correlated with GM density in the right posterior HC. We further showed that ET users compared to never users have greater GM concentration in the left nucleus accumbens (NAcc) when compared to never users and that this effect is modulated by age at menarche and age at menopause. Investigating sex differences, we found that women have larger right NAcc, hippocampi, inferior temporal gyri and prefrontal cortices compared to men. These results demonstrate that estrogens play a role in brain aging outside of the HC, prompting future studies to investigate the effect of estrogen exposure on the nucleus accumbens and its function.

Overall, both study results strongly suggest that endogenous and exogenous estrogen exposures have modulatory neuroprotective effect on the aging brain.
Implications

The future of HT being at a crossroad, this thesis provides several indications for new studies assessing the differential impact of lifelong estrogen exposure and both current and past use of HT on the brain.

We offer a questionnaire inventorying different indices of lifelong estrogen exposure that have been previously reported to have an impact on the brain and/or the behaviour. We cover variables from the perinatal period to the postmenopausal years. This questionnaire can be of use not only in the neuroscience field but may also be of interest in the oncology and cardiovascular research fields among others.

The reliability assessment results, put in the context of the current controversy about the effect of estrogens on the brain, warn the scientific community about the pitfalls of self-reported measures. Scientists interested in the impact of these indices on one’s health should be certain about the reliability and, even more importantly, validity of self-reported information before pushing further their quest in the core of their interrogations. This becomes even more of an issue when older individuals are asked to provide information about their past since in our sample the recall of older women was the least reliable across a four year interval.

Indeed, our results also demonstrate that age at menopause and age at HT initiation were the two less reliable recalled indices, warning about the accuracy of these crucial indices. Since it was these two indices as opposed to age at first menstruation for example, suggest that reliability is perhaps not so much affected by the time elapsed between the actual event and the moment of the recall. Rather, the decrease in reliability might be related to the menopausal period itself. Conversely to age at menarche, age at menopause might be more difficult to remember since the years prior menopause are characterized by cycle irregularities whereas menarche is a single isolated and most of the time painful and emotional event. Moreover, it might even be more difficult to remember precisely age at menopause because menopause is in fact the absence of an event for a year, not an isolated event like menarche or even pregnancy. The reliability of age at HT initiation might suffer as a consequence, since in most cases it occurs at the same age as age at menopause. Based on our results and the known biology of menopause we further advice on making an extra effort to collect the most accurate data possible when precise information about the menopausal years is needed. As suggested in the article,
information from physician/pharmacy records might become useful to corroborate self-reported information.

In our attempt to characterize why women do not arrive at menopause with an equivalent susceptibility to the effects of ET on the brain, we demonstrated that lifelong estrogen exposure was an important variable to consider. In both neuroimaging studies we were able to support previous preliminary findings by Eberling et al.(2003) demonstrating that post-menopausal HT users have larger HC volume compared to non-users and men. However, we were the first to show a time-dependent effect of ET use on the hippocampus. In an attempt to investigate this duration effect further, we called back our participants 3 years after they were first scanned. Unfortunately, we were only able to rescan 4 ET users, 3 past users, 3 never users, and 3 men (see Table 1). All the participants that were reached accepted to participate but most of the postmenopausal women were unreachable.

Table 1 Demographic information of the longitudinal HC results

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Education</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET users</td>
<td>62.3</td>
<td>14.0</td>
<td>25.0</td>
</tr>
<tr>
<td>n = 4</td>
<td>± 7.4</td>
<td>± 2.9</td>
<td>± 5.9</td>
</tr>
<tr>
<td>Past users</td>
<td>64.0</td>
<td>14.7</td>
<td>25.0</td>
</tr>
<tr>
<td>n = 3</td>
<td>± 3.6</td>
<td>± 5.0</td>
<td>± 2.6</td>
</tr>
<tr>
<td>Never users</td>
<td>60.0</td>
<td>16.7</td>
<td>27.3</td>
</tr>
<tr>
<td>n = 3</td>
<td>± 7.2</td>
<td>± 2.3</td>
<td>± 2.3</td>
</tr>
<tr>
<td>Men</td>
<td>59.7</td>
<td>14.5</td>
<td>27.0</td>
</tr>
<tr>
<td>n = 3</td>
<td>± 3.2</td>
<td>± 2.1</td>
<td>± 1.7</td>
</tr>
</tbody>
</table>

Interestingly, ET users demonstrated a HC volume decline of about 3.7 %, the past users declined minimally, the never users stayed stable over time and men showed a 2.8% decline from time 1 to time 2 (see Figure 1).
Figure 1: mean HC volumes per group on time 1 and 2

Even if the sample size is too small to allow statistical analysis, the visual examination of these longitudinal data add to the previously demonstrated time-dependent effect of ET on HC volume as well as the age-related decline in HC volume observed in men. Moreover, the 4 ET users had respectively used ET for 11, 12, 17.5, and 30 years (mean 17.7) all exceeding the hypothesised treatment duration threshold of about 10-11 years of exposure for the beneficial effects of HT on cognitive function (MacLennan, Henderson et al. 2006) and the brain (Erickson, Colcombe et al. 2007). These preliminary results further support the idea that long-term ET use is increasingly detrimental whereas short-term use might preserve HC volume to an optimal level.

This brings us to the unanswered question of what is the functional significance of having larger or smaller HC volume. In an attempt to disentangle the current literature, Van Petten (Van Petten 2004) did a review along with a meta-analysis including studies investigating the link between HC volume and memory function and this, across the lifespan. Based on these results, it was conclude that the model arguing that «bigger is always better» is weakly supported. However, the view that normal size HC will support normal function until a certain loss of tissue occur provides a better framework to explain volume decreases due to normal aging accompanied by memory related decline.

It is well known that structures of the temporal lobe such as the entorhinal cortex and the HC are implicated in memory function but they are also of great importance in the progression of Alzheimer disease (AD). AD being characterized initially by deficits in forming new memories as well as concomitant temporal lobe
atrophy, particularly of the HC and entorhinal cortex. In healthy populations, results tend to demonstrate that the anterior part of the HC is linked to encoding new materiel whereas the posterior part would be more involved in retrieval processes (Lepage, Habib et al. 1998). Recently, a study assessed whether the different volumes of HC sub-regions were associated with verbal memory performance. They demonstrated that healthy older adults with larger HC heads were able to encode and retrieve more words (Hackert, den Heijer et al. 2002). Following the cognitive continuum, it was observed in individuals at risk to develop AD with age-associated memory impairment (AAMI), that the volume loss was greater in the right HC head (Mega, Small et al. 2002). This argues in favour of both the anterior and the right-greater-than-left location of HC atrophy in AD previously described (Jack, Petersen et al. 1997). Interestingly, ET use has been repeatedly reported to decrease the risk or delay the onset of AD but no consensus exists on the possible underlying mechanisms (Henderson 1997; Fillit 2002). The observed greater HC volume and grey matter density in the right HC head and entorhinal cortex in ET users might provide support for the latter idea of estrogen neuroprotection on memory function and against early AD assaults following menopause.

The functional significance of structural difference being of important nature we included neuropsychological assessments in a subset of our sample. We tested memory function using neuropsychological tasks known to demonstrate estrogen effects on cognition and also targeting hippocampal functions. We thus chose to use a story recall, a standardized word list, the digit span, and a standardized visual memory task. We gathered data from 13 ET users, 15 non users (including 6 past HT users and 9 never users merged for sack of clarity) and 15 men equivalent for age, education and body mass index (BMI) (see Table 2).
Table 2 Demographic information of the memory study

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Education</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET users</td>
<td>57.3</td>
<td>13.5</td>
<td>23.4</td>
</tr>
<tr>
<td>n = 13</td>
<td>± 5.1</td>
<td>± 2.5</td>
<td>± 3.9</td>
</tr>
<tr>
<td>Non users</td>
<td>58.8</td>
<td>13.7</td>
<td>23.7</td>
</tr>
<tr>
<td>n = 15</td>
<td>± 6.9</td>
<td>± 3.3</td>
<td>± 3.1</td>
</tr>
<tr>
<td>Men</td>
<td>55.7</td>
<td>14.4</td>
<td>26.3</td>
</tr>
<tr>
<td>n = 15</td>
<td>± 4.0</td>
<td>± 2.0</td>
<td>± 3.9</td>
</tr>
</tbody>
</table>

The analysis of variance assessing group difference for the memory scores gave rise to non-significant findings (all p>0.1). Moreover, HC volume and memory scores did not significantly correlate among the whole group or inside each group (all p>0.05).

Based on our hypothesis that menopause might be the missing link between neuroimaging findings (see Figure 3, Chapter 1) we first recruited middle aged subjects aged between 50 to 65 years. A risk was taken there since studies that find the positive effect of HT on memory functions usually include women older than 65 years of age. Therefore, our sample might have been too young and well functioning to demonstrate the impact of ET on memory functions. These results point toward a threshold effect arguing in favour of the idea describe earlier that normal size HC will support normal function until a certain loss of tissue occur. It can be hypothesized that memory decline can only be observed later in life when a certain HC volume threshold is reached. ET users having larger HC volume might only demonstrate this cognitive decline later in life and/or after certain duration of ET use explaining the mixed memory results in the field. However, in order to ascertain this hypothesis future study should measure HC volume and test memory function in postmenopausal women of different treatment duration and age (wider age range from 50 to 80 for example).

Another interesting aspect of this thesis is the unexpected results of the VBM analysis. Both animal and human studies focused their attention on the effect of estrogens on the hippocampus for obvious reasons. However, using a whole brain analysis technique we demonstrated that ET might impact on the striatum (NAcc). This brought us to question the interactions between estrogens and neurotransmitter
systems such as dopamine (see Chapter 4). Several evidence from the animal literature exist on the protective effect of estrogen on the nigrostriatal dopaminergic pathway involved in motor function (Dluzen and Horstink 2003) and the possible involvement of estrogen in the mesolimbic and mesocortical pathways involved in cognitive activities, affective state, motivated behaviour, and reward processing (Becker 1999; Paredes and Agmo 2004). Animal studies showed that there are sex difference and gonadal modulation of the dopamine activity in the striatum and nucleus accumbens (Becker 1999).

However, until recently few studies investigated these interactions in humans. One recent study investigated the influence of estrogen and progesterone on the human reward system using functional MRI (Dreher, Schmidt et al. 2007). Briefly, they studied healthy cyclic women across their menstrual cycle and men using a monetary reward paradigm in a within-subjects fMRI design. Their results support the idea that the reward circuit is sexually dimorphic and influenced by gonadal hormones (Dreher, Schmidt et al. 2007). These exciting results along with our VBM results pave the way to human studies interested in the influence of estrogen (both current level and exposure indices) on striatal regions and dopamine interactions.

Finally, even if this thesis does not have a direct impact on women's life, it is part of a larger effort to characterize the effects of estrogens and HT on the brain and its functions that is crucial for menopausal women. Based on studies investigating the effect of lifelong estrogen exposure among other things, in this new era of « Different women, different needs », we will hopefully be able in the near future to prescribe the right HT to the right women, at the right time, and for the right duration.

Limitations

One of the main limitations of our studies is the small sample size. Our experimental design being a neuroimaging one, we were tied by budget constraints. In order to investigate the functional significance of each and every one of the lifelong estrogen indices larger samples are required. However, even with this limitation we were able to uncover the importance of HT duration among other things.

Another limitation preventing us to generalize our results might reside in the choice we made while designing the study. Based on previous study design, it was
important for us to isolate the effect of estrogens from the one of progesterone and/or its interaction with estrogens. However, by excluding EPT users from our sample of current users as a consequence, we ended up with a large proportion of surgically induced menopausal women (8/16). Even if no difference in brain volumes was observed between these two group of women (see chapter 3), there is the possibility that having an abrupt menopause (surgical) as opposed to a gradual one (natural) does not have the same consequence on the brain and memory functions (Henderson and Sherwin 2007). In the same line of thought, ET preparations might also introduce potential confounds since both conjugated equine estrogen (CEE) and estradiol users were included.

Retrospectively, we have shown that the self-reported nature of our variables are not the best when it comes to age at menopause and age at HT initiation for the previously described reasons. However, age at menarche and HT duration (see Chapter 3 & 4), crucial indices in our results, demonstrated an excellent reliability.

Finally, these studies lack functional significance. Our goal was to investigate estrogens effect on brain structures and we were successful in doing so. However, we can minimally conclude about the functional significance of having larger hippocampal volume and we cannot conclude anything about having a denser nucleus accumbens. Future studies replicating our results should include memory testing and symptoms assessments among other things in a wide age spectrum.

Future directions

To this date several questions remain and need further investigations in order to better understand the impact of estrogens on the brain. Potential future directions are presented.

Longitudinal study

Regarding the effect of ET on HC volume a question of importance remains unanswered: Does ET increase HC volume or does it preserved it against age-related decline following menopause? We still do not know if the drop in estrogens following menopause leads to a decrease in HC volume that can be prevented by ET use. A longitudinal design could help elucidate this matter. Recruiting a large sample
of premenopausal women and following them several years into their menopausal years could clarify this.

Furthermore, this design could also clarify a question of great interest: Is there a window of opportunity for the positive effects of estrogen on the brain? Since some women engaged on this hypothetic study would decide to take HT therapy, variables such as time since menopause and HT duration effect could be investigated.

More importantly, lifelong estrogen exposure indices could be investigated. Women would provide information about their reproductive years. Age at menopause and HT initiation and cessation would not cause a problem since premenopausal women who would be recruited.

HT and neurotransmitters

With the advent of new neuroimaging techniques it would hopefully be more frequent in the future to assess the interactions of estrogens with neurotransmitters. Study results in human and animal along with our VBM results brought us back to the hypothesis that estrogens might interact with neurotransmitters such as dopamine. With this in mind it would be interesting to test if in post-menopausal women the restoration of sexual appetite brought on by ET is mediated by the dopamine-enhancing effect of estrogen in the NAcc. Using PET and raclopride as a ligand, it would be interesting to see if the use of ET is linked with dynamic changes in dopamine concentration in the striatum. Menopausal symptoms should be assessed to see if it correlates with restoration of sexual function. Again an ideal design would be to scan symptomatic women prior to the use of ET and 6 months to a year later.

Conclusion

The goal of the current thesis was to propose a new look at the effects of estrogens on the brain by introducing new measures. We first assessed the reliability of indices of lifelong endogenous and exogenous estrogen exposure. Secondly, volumetric analyses of regions of interest in the brain (hippocampus and amygdala) as well as global automated analysis using voxel-based morphometry (VBM) were performed in order to assess whether current ET use as well as estrogen exposure throughout life can predict age-related structural changes in the aging brain of postmenopausal women. Results demonstrated that effectively, lifelong estrogen
exposure should be taken into account when studying the effect of estrogens on the brain and its functions. More importantly, this thesis gave rise to many more questions paving the way to a more detailed understanding of hormones and the brain in aging.
Chapter 6:

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Chapter 7:
Appendices
Appendix 1:

Questionnaire «Indices of Estrogen Exposure»
Indices of estrogen exposure

ID: ________________________  Date of Birth: ______________

Many studies attempt to understand the influence of sex hormones on our physical and mental health. In this questionnaire we focus on determining the variations of estrogen over the course of one's life. Many factors can affect endogenous estrogen levels. For example, perinatal estrogen exposure, the menstrual cycle, pregnancies, menopause and also life habits. Other factors, like synthetic hormones such as the contraceptive pill or hormone therapy after menopause, vary the level of hormones in the body. Therefore, this questionnaire will ask some questions relating to factors that may influence estrogen levels in order to determine the impact of this hormone on one's health.

The questionnaire is divided in 6 sections; please respond to the following questions to the best of your knowledge with as many details as you can remember.

1) **Perinatal estrogen exposure**

What is your birth order in your family: _______________

To the best of your knowledge, do you remember?

- Your weight at birth: __________
- Your size at birth: __________
- If you were premature (born more than 4 weeks prior the due date) (y/n): ______
  - If yes, at how many weeks were you born: __________ weeks

Please attach a photocopy of your hands in order for us to calculate the ratio between your 2\textsuperscript{nd} (index) and 4\textsuperscript{th} finger (ring finger) as an indirect measure of perinatal estrogen exposure.

<table>
<thead>
<tr>
<th></th>
<th>Left hand</th>
<th>Right hand</th>
</tr>
</thead>
<tbody>
<tr>
<td>2\textsuperscript{nd} digit length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4\textsuperscript{th} digit length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
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</tbody>
</table>
2) **Menstrual cycle**

At what age did you have your first menstruation? _________

Over the course of your life would you consider your periods to be regular, i.e. a cycle that lasts between 24 and 35 days? (y/n) _________

If not, please explain what you’re approximate cycle was like.

________________________________________________________________________

________________________________________________________________________

Did you ever go through periods where you stopped menstruating (besides menopause and pregnancies)? (y/n) _________

If yes, do you remember how many months you skipped and do you know the reason why this may have occurred?

________________________________________________________________________

________________________________________________________________________

Have you ever taken the contraceptive pill? (y/n) _________

If yes, please fill out the table below, including every prescription that you have ever had, as precisely as you can remember.

<table>
<thead>
<tr>
<th><strong>Contraceptive pill brand</strong> (Ex.: Triphasil, Ortho-cyclen, ...)</th>
<th><strong>Age at beginning of use</strong></th>
<th><strong>Age at end of use</strong></th>
<th><strong>Duration of use</strong></th>
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</thead>
<tbody>
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</tbody>
</table>
3) **Motherhood**

Do you have any children? (y/n) _________

If yes, please fill out the following table.

<table>
<thead>
<tr>
<th>Child</th>
<th>Date of birth</th>
<th>Your age</th>
<th>Is this child adopted? (y/n)</th>
<th>Did you breast feed? (y/n)</th>
<th>If yes, for how many months?</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7</td>
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</table>

Did you have any interruptions of your pregnancies (abortions, stillbirths or miscarriages)? (y/n) _________

If yes, please specify how many and the duration of each pregnancy using the following table.

<table>
<thead>
<tr>
<th>Pregnancy</th>
<th>Your age</th>
<th>Duration of pregnancy (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
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<td>2</td>
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<td>3</td>
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<td>5</td>
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</tbody>
</table>
4) Menopause

The fourth and fifth sections are aimed at determining information about the menopausal phase of your life. Menopause is the point in time during which you stop ovulating permanently. Before menopause most women undergo a phase of fluctuation during which their menstrual cycles are irregular.

Approximately, when did your menstruations start becoming irregular, if applicable? (Ex: around 10 months prior to my last period.)

At what age did you have your last menstruation? ______________________

Did you encounter menopausal symptoms? (Ex: hot flashes, insomnia, sexual related trouble, etc) (y/n) ________

If yes, please make a list of your menopausal symptoms?

________________________________________________________________________

________________________________________________________________________

Have you encountered menopause naturally or through a surgery, please specify?

☐ Natural

☐ Surgical

☐ Hysterectomy (removal of the uterus)
☐ Ovarectomy (removal of the ovaries)
☐ Both
☐ Don't know

If your menopause was surgically induced (i.e. ovarectomy and/or hysterectomy), if you know the reasons for this intervention please tell us to the best of your knowledge.

________________________________________________________________________

At what age did you get the surgery? ______________________
5) Hormone therapy

Have you ever taken hormone therapy? (y/n) _____________

Could you explain what have motivated your decision to take (or not take) hormone therapy?

________________________________________________________________________

If you have ever taken or currently take hormone therapy, could you please fill out the table below, including every prescription that you have ever had, as precisely as you can remember?

<table>
<thead>
<tr>
<th>Hormone therapy brand and dosage (Ex.: Premarin 625mg/day and Provera 10mg/day)</th>
<th>Age at start of treatment</th>
<th>Age at end of treatment</th>
<th>Treatment duration</th>
</tr>
</thead>
<tbody>
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<td>□ pill □ patch □ gel</td>
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<td>□ pill □ patch □ gel</td>
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</table>

If you have ever taken or are currently taking hormone therapy, could you please use the scale below to evaluate your feeling of well-being during the treatment compared to when you were not taking hormones.

1 worse  2 a bit worse  3 same  4 a bit better  5 better
6) **Life Habits**

Do you currently smoke cigarettes or have you smoked in the past? (y/n) ______

If **yes**, please specify your daily consumption.

Between 20 and 30 years of age? ________ Cigarettes per day
Between 30 and 40 years of age? ________ Cigarettes per day
Between 40 and 50 years of age? ________ Cigarettes per day
Between 50 and 60 years of age? ________ Cigarettes per day
Between 60 and 70 years of age? ________ Cigarettes per day
Between 70 and 80 years of age? ________ Cigarettes per day
Over 80 years of age? ______________ Cigarettes per day

Could you tell us your current weight and height?

Could you please tell us your weight and height between these time periods?

Between 20 and 30 years of age? ________
Between 30 and 40 years of age? ________
Between 40 and 50 years of age? ________
Between 50 and 60 years of age? ________
Between 60 and 70 years of age? ________
Between 70 and 80 years of age? ________
Over 80 years of age? ______________

How would you rate your fitness level thinking of the number of hours you spent playing a sport or exercising using the numbers below?

1. 1-2 hours per month
2. less than 4 hours per month
3. at least an 1 hour per week
4. about 2 hours per week
5. more than 2 hours per week

Between 20 and 30 years of age? 1 2 3 4 5 _____________
Between 30 and 40 years of age? 1 2 3 4 5 _____________
Between 40 and 50 years of age? 1 2 3 4 5 _____________
Between 50 and 60 years of age? 1 2 3 4 5 _____________
Between 60 and 70 years of age? 1 2 3 4 5 _____________
Between 70 and 80 years of age? 1 2 3 4 5 _____________
Over 80 years of age? 1 2 3 4 5 _____________

Thank you for your participation

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Appendix 2:

Research Ethic Board Certificates