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TANELI HEIKKINEN

Cognitive Effects of Estrogen in Ovariectomized, Aged and Transgenic Mice Modeling Alzheimer's Disease

Doctoral dissertation

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Department of Neurology, University of Kuopio
Department of Neurology, Kuopio University Hospital

Distributor:
Department of Neurology
University of Kuopio
P.O. Box 1627
FI-70211 Kuopio
FINLAND
Tel. +358 17 162 682
Fax +358 17 162 048

Author's address:
Department of Neurology
University of Kuopio
P.O. Box 1627
FI-70211 Kuopio
FINLAND
Tel. +358 17 162 518
Fax +358 17 162 048
E-mail: Taneli.Heikkinen@uku.fi

Supervisors:
Professor Heikki Tanila, M.D., Ph.D.
Department of Neurobiology
A. I. Virtanen Institute
University of Kuopio

Jukka Puoliväli, Ph.D.
Cerebricon Ltd.
Kuopio

Reviewers:
Professor Pirkko Härkönen, M.D., Ph.D.
Department of Laboratory Medicine, Tumor Biology
Lund University
Malmö, Sweden

Professor Etan Markus, Ph.D.
Department of Psychology
University of Connecticut, USA

Opponent:
Docent Tomi Taira, Ph.D.
Neuroscience Center and Department of Biosciences
University of Helsinki

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ABSTRACT

Alzheimer's disease (AD), usually occurring after 60 years of age, is the most common form of dementia and its incidence is higher in women than men. It has been suggested that the decline in the levels of gonadal hormones in postmenopausal women might contribute to this gender difference. In the 1990's, it was claimed that the estrogen replacement therapy (ERT) given to postmenopausal women perhaps would be able to alleviate the cognitive symptoms of AD and delay the onset of AD, even prevent the disease. The majority of the epidemiological studies on ERT at that time and the early 2000's did indicate that ERT was able to improve cognitive abilities in postmenopausal women and decrease the incidence of AD. However, some recent studies have challenged this view and even suggested that ERT could increase the prevalence of AD.

While the contribution of ERT on cognitive performance and pathogenesis of AD in women is still not clear, animal models provide a means to examine the basic effects of estrogen on brain functions. Indeed, a number of animal studies using different methodological approaches have shown that estrogen modulates neuronal morphology in the hippocampus, a brain area important for certain forms of memory and also the locus of extensive neuronal damage in AD. Estrogen has also been reported to enhance synaptic functions in the hippocampus and modulates several neurotransmitter systems. Further, in cell cultures, estrogen has been shown to decrease the amount of beta amyloid (Aβ), a major histopathological hallmark of AD.

In this study, we wanted to examine the effects of ERT on cognitive performance in female mice in two different memory tasks, the radial arm maze (RAM) and the T-maze. The postmenopausal state was mimicked by ovariectomy (OVX). First, we assessed the effects of ERT in young adult animals, in which we compared two different modes of treatment, tonic and phasic. Our intention was to examine if alternations in acetylcholine and monoamine metabolism or the mRNA levels of estrogen receptors or aromatase enzyme in the hippocampus might contribute to the possible effects of ERT on cognition. Second, we explored the effects of OVX and ERT in aged mice in the same memory tasks. Finally, the same question was assessed in transgenic mice (AP mice) with an AD-like pathology, carrying mutations in two genes encoding for amyloid precursor protein (APP) and presenilin 1 (PS1). These AP mice display a progressive accumulation of Aβ and exhibit the formation of amyloid plaques, another hallmark of AD. Therefore, we also wished to determine whether ERT could affect the Aβ metabolism in these mice.

In young adult OVX mice, long-term tonic ERT improved the acquisition of RAM and T-maze. The same treatment induced changes in serotonin turnover and mRNA levels of estrogen receptor alpha and aromatase enzyme, suggesting that these changes may contribute to the observed behavioral effects. The effects of ERT on cognitive performance in aged OVX mice after long-term estrogen deprivation were similar, but notably smaller. Also, in OVX AP mice of different ages, the effects of ERT were strikingly similar, but clearly not dependent on Aβ pathology. In summary, our results suggest that long-term ERT may have beneficial effects on some forms of memory in adult, aged and transgenic mice with AD-like pathology, if started sufficiently early after estrogen deprivation. In contrast, these results do not support the view that ERT could slow down or prevent the underlying amyloid pathology.

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Medical Subject Headings: Alzheimer Disease; Alzheimer Disease/epidemiology; Estrogen Replacement Therapy; Estrogens; Female; Hippocampus; Learning; Memory; Mice; Mice, Transgenic; Models, Animal; Ovariectomy

If you don't live it,
it won't come out of your horn.
Charlie Parker

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Kuopio, May 2006

Taneli Heikkinen

ABBREVIATIONS

Aβ	beta amyloid
AD	Alzheimer's disease
AF	activating function
AP	APPswe + PS1(A246E)
APP	amyloid precursor protein
CHD	coronary heart disease
CEE	conjugated equine estradiol
ChAT	choline acetyltransferase
CNS	central nervous system
DA	dopamine
DMP	delayed matching to position
DOPAC	dihydroxyphenylacetic acid
ERE	estrogen receptor response element
ER	estrogen receptor
ERT	estrogen replacement therapy
5-HT	5-hydroxytryptamine (serotonin)
5-HIAA	5-hydroxyindoleacetic acid
FSH	follicle stimulating hormone
HRT	hormone replacement therapy
LTM	long-term memory
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
MT	menopausal transition
NA	noradrenaline
NMDA	N-methyl D-aspartate
OVX	ovariectomy
PS1	presenilin 1
RAM	radial arm maze
SHAM	sham-operated

STM	short-term memory
WHI	Women’s Health Initiative
WM	water maze

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals I-IV.

I. Heikkinen T., Puoliväli J., Liu L., Rissanen A., Tanila H. Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice. Hormones and Behavior 41, 22-32 (2002).

II. Iivonen S.*, Heikkinen T.*, Puoliväli J., Helisalmi S., Hiltunen M., Soininen H., Tanila H. Effects of estradiol on spatial learning, hippocampal cytochrome P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice. Neuroscience 137, 1143–1152 (2006).

III. Heikkinen T., Puoliväli J., Tanila H. Effects of long-term ovariectomy and estrogen treatment on maze learning in aged mice. Exp. Gerontol. 39, 1277-1283 (2004).

IV. Heikkinen T., Kalesnykas G., Rissanen A., Tapiola T., Iivonen S., Wang J., Chaudhuri J., Tanila H., Miettinen R., Puoliväli J. Estrogen treatment improves spatial learning in APP+PS1 mice but does not affect beta amyloid accumulation and plaque formation. Exp. Neurology 187, 105-117 (2004).

* both authors contributed equally to this work

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1. INTRODUCTION

When this study was started at the end of the 1990's, estrogen replacement therapy (ERT) was being promoted as a potential therapeutic strategy for delaying the onset or even preventing Alzheimer's disease (AD) in postmenopausal women. This idea was supported by several studies suggesting that ERT was associated with reduced risk for AD and cognitive decline in postmenopausal women (Baldereschi et al. 1998, Paganini-Hill and Henderson 1994, Paganini-Hill and Henderson 1996, Resnick et al. 1997, Tang et al. 1996, Waring et al. 1999). However, this claim has been strongly questioned by some recent studies, especially by the large Women's Health Initiative (WHI) study which reported that ERT actually increased the incidence of dementia in addition to having other detrimental physiological effects in postmenopausal women (Shumaker et al. 2003). It has to be stated that both the earlier studies describing beneficial effects with ERT and the WHI study suffer from methodological pitfalls that make it impossible to take a definitive stand on this subject.

During the last two decades, studies with experimental animals have yielded a considerable amount of information in favor of the beneficial effects of estrogen on cognition. Perhaps the strongest evidence is the observation that estrogen is capable of increasing the density of the dendritic spines of pyramidal neurons in the female rat hippocampus (Gould et al. 1990, Woolley et al. 1990), a brain area crucial for several memory types. Estrogen has also been reported to increase the activity of the cholinergic system (Luine et al. 1975) which is severely affected in AD, and it also seems to have many neurotrophic effects in the brain (Toran-Allerand et al. 1999).

In a variety of animal studies, conducted mainly with young or middle-aged rats and mice, in which the postmenopausal state has been mimicked by ovariectomy (OVX), estrogen treatment has been shown to improve some forms of learning and memory, most often spatial working memory (Bimonte and Denenberg 1999, Daniel et al. 1997, Daniel and Dohanich 2001, Fader et al. 1999, Miller et al. 1999). Furthermore, estrogen treatment appears to be effective in improving learning and memory also in aged female rodents (Frick et al. 2002, Gibbs 2000b, Markham et al. 2002, Markowska and

Savonenko 2002). However, some other studies have reported no treatment effect (Luine and Rodriguez 1994, Singh et al. 1994) or even impaired spatial learning (Fugger et al. 1998) in estrogen-treated OVX rodents. Although estrogen might not be effective in the treatment of established AD, it could be able to slow down the underlying pathology during its early stage. One possible mechanism could be the inhibition of brain beta amyloid (Aβ) accumulation. Plaques rich in Aβ are a central hallmark of AD and indeed, in cell cultures, estrogen has been shown to reduce the formation of Aβ (Jaffe et al. 1994, Xu et al. 1998).

In this study, we wanted to evaluate the effects of ERT on cognitive performance in young adult and aged female mice. We also wished to determine if changes in hippocampal neurotransmitters and estrogen receptor contents would be affected by estrogen treatment. In addition, we were interested to examine if estrogen could have any impact on learning and memory in transgenic mice with AD-like pathology and also, if this pathology could be affected by estrogen treatment.

2. REVIEW OF LITERATURE

2.1. Estrogen and general physiology

2.1.1. Structure and biosynthesis of estrogen

Estrogens are lipid-based reproductive hormones belonging to the group of steroids, a large group of molecules, all derived from a sterol precursor, cholesterol. There are three different forms of estrogens: estradiol, estriol and estrone. Estradiol, also termed as 17β-estradiol or E2, is the most potent of the estrogens and whenever estrogen is mentioned in its singular form throughout this thesis, this refers to estradiol. In general, estriol has approximately 10 % of the physiological potency of estradiol while estrone has only about 1 % of estradiol's potency (Johnson and Everitt 1995). In females, estrogen is mainly synthesized by the ovaries and during gestation also by the fetoplacental unit. Estrogen is released into the blood circulation from these sites. In addition, local estrogen biosynthesis occurs in various tissues throughout the body, and this occurs in both sexes (Simpson et al. 1999). Regardless of where the estrogen biosynthesis takes place, the biochemical synthetic pathways are usually very similar.

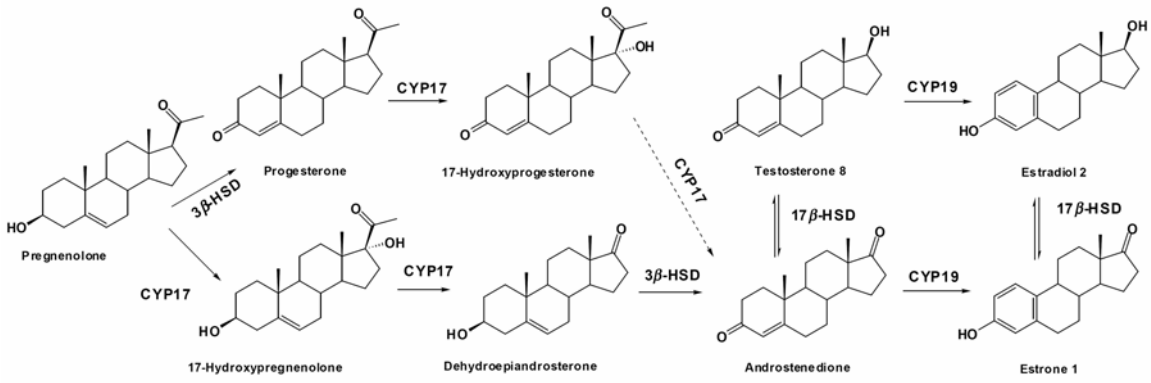

Figure 1. Estrogen biosynthesis.

The cholesterol needed for estrogen synthesis is mostly produced by the liver but also can originate from other tissues. Cholesterol is converted to pregnenolone, this process being catalyzed by a P-450 enzyme. From pregnenolone, the estrogen can be synthesized via two different pathways: conversion of pregnenolone first either to progesterone or to 17α-hydroxypregnenolone (Fig. 1). Finally, the estrogens are formed

either from androstenedione (for estrone) or from testosterone (for estradiol) via the help of another P-450 enzyme, the aromatase complex enzyme (P-450Arom).

2.1.2. Estrogen's mechanisms of action and estrogen receptors

Estrogen exerts its actions in cells and thereby tissues by binding to certain structures in the cell membranes or by activating intracellular receptors that activate specific signaling pathways. To date two such receptors that can be found in different tissues throughout the body have been characterized in both genders (Kuiper et al. 1998), estrogen receptor alpha (ERα) and beta (ERβ). The latter receptor was first identified in rat (*Rattus norvegicus*) prostate in 1995 and was thus named ERβ to differentiate it from the so called classical estrogen receptor, which ever since has been called ERα (Kuiper et al. 1996).

Both ERα and ERβ belong to the family of nuclear receptors which are ligand-regulated transcription factors (Pettersson and Gustafsson 2001). ERs regulate gene expression by binding to specific ER response elements (ERE) or via interactions between other transcription factors (Paech et al. 1997). ERα and ERβ are very similar in the sequence of their DNA-binding domains and therefore their affinity and specificity of binding EREs are also similar (Matthews and Gustafsson 2003). Their ligand binding domains are significantly different, but they bind to estrogen with nearly identical affinities (Matthews and Gustafsson 2003). The activating functions of ERs are mediated by two different transcription activating functions (AFs): an N-terminal activating function (AF-1) that is independent of ligand activation and a ligand-binding domain (LBD) – based ligand-dependent activating function (AF-2) (Nilsson et al. 2001). The AFs are responsible for the estrogen-mediated transcription and the promoter- and cell-specificity. Although both ER subtypes have highly similar mechanisms of action, there are differences in their transcriptional functions and therefore they might regulate different cellular pathways (Matthews and Gustafsson 2003).

The two different receptor subtypes differ also in tissue specificity and functional characteristics. Although both receptors can be localized more or less throughout the body, one of the two is often predominant in any given tissue. ERα is expressed to a

high or moderate degree in uterus, testis, pituitary gland, ovaries, kidneys and epididymis, whereas ERβ is abundant in prostate, ovaries, lungs, bladder, brain, bones, uterus and testis (Kuiper et al. 1998). The functional differences between the two subtypes are not fully understood. For example, if ERα is inactivated, the uterus shows very little response to estrogen (Korach et al. 2003), and when there is no estrogen stimulation, the uterus does not grow (Simpson 2004). When ERβ is inactivated, the uterus is abnormally large i.e. the uterine response to estrogen is intensified (Weihua et al. 2000). Therefore it seems that since ERα is the dominant receptor in uterus, loss of ERβ would be unlikely to affect the uterine response to estrogen stimulation. In the tissues in which ERβ is dominant, such as the ovary and certain parts of the brain, estrogen is mainly responsible for maintenance of structure or function of the tissue, but does not have a proliferating effect as it does in the uterus. Further, an experiment using cell lines has shown that, in the presence of ERα, estrogen elicits proliferation, but in the presence of ERβ, it inhibits proliferation (Strom et al. 2004); thus, the estrogenic effect seems to depend significantly on which receptor subtype is activated.

The discovery of the ERβ in 1995 led to speculation about other mechanisms via which estrogen could exert its cellular effects. Indeed, subsequently many such alternative mechanisms have been found: different estrogen-binding proteins, alternative splicing variants of classical ERs and presumably even new genes. One such receptor, called as ERχ, has been identified, but so far only in a teleost fish (Hawkins et al. 2000). Furthermore, the rapid actions of estrogen (Kelly and Levin 2001) that clearly could not be achieved via activation of the nuclear ERs and subsequent gene transcription are evidence in favor of non-genomic pathways. One of these mechanisms could be ERs close to the plasma membrane. Indeed, both ERα and ERβ in addition to the ERα variant ER-46, have been localized to the plasma membrane (Chambliss et al. 2002, Li et al. 2003b, Razandi et al. 2002). Additionally, novel ERs have been found close to the plasma membrane that do not belong to ERα or ERβ (Nadal et al. 2000) or G protein coupled receptors (Filardo et al. 2000). And so that story should not remain too simple, a recent report by Toran-Allerand and her co-workers (2002) has described yet another different receptor, tentatively named as ER-X, a developmentally regulated, plasma membrane-associated receptor. The characteristics and functional roles of many of the

novel ERs (and partly of the classical ERα and ERβ) are only beginning to be understood.

2.1.3. Regulation of estrogen secretion

Estrogen has a self-evident role in reproduction and other sexual functions. The female reproductive status is regulated by the ovarian cycle, menstrual cycle in women and the estrous cycle in animals. A complete ovarian cycle is the interval between two consecutive ovulations. In humans, the menstrual cycle usually starts to appear at the time of puberty, around 11-15 years of age. During each cycle, one of the primordial follicles resting in the ovaries matures and develops into the so called Graafian follicle. The estrogens are secreted by the Graafian follicles and thus the blood estrogen levels are high during the phase preceding ovulation (the follicular phase). The mature follicle then bursts and the egg cell is released at the half way point of the cycle (about 14^{th} day) and finds its way to the oviduct where possible fertilization takes place. At the same location where the follicle was situated the yellow body of the ovary (*corpus luteum*) is formed and this starts to secrete both estrogens and progesterone. In fact, about 95 % of all estradiol secreted during the cycle is derived from the *corpus luteum*. The postovulatory period (luteal phase) is still dominated by progesterone secreted by the *corpus luteum*. If the egg cell does not become fertilized, the *corpus luteum* undergoes atrophy, causing the uterine mucosa to become thinner and weaker and ultimately to shed its lining prior to the start of a new menstrual cycle.

Different species of rodents have quite different estrous cycles, but the cycles of rats and mice are very similar. In mice (*Mus musculus*) the time of puberty varies extensively, depending on the strain and rate of growth. The onset of puberty is determined by the maturation of egg cells in the ovaries. Usually this happens around days 28-49 after birth, with the first estrous cycle being observed one or two days later. A female mouse is at the peak of its fertility between three and ten months of age (Hafez 1970). A laboratory mouse is a polyestrous animal, meaning that it has several estrous cycles during the year and the cycles follow one another without a break. The same is not true for mice living in wild, since their breeding seasons are usually concentrated in the period spring to autumn. However, irrespective of whether the

mouse lives in a laboratory or in the wild, the estrous cycle lasts for four to five days. The estrous cycle of a mouse is divided into four different stages: proestrus, estrus, metestrus and diestrus in that chronological order. The basic physiology of the estrous cycle of the mouse is very similar to the menstrual cycle of human females, except for the duration (see Fig. 2.). One major difference between the two species is that in mouse the *corpus luteum* is inactive after the follicle has burst unless mechanical stimulation of the uterine cervix occurs, which would subsequently be followed by a plug being formed from the liquids ejected by the mating male. This plug would then activate the *corpus luteum* until the embryo was attached. If the fertilization does occur, then the *corpus luteum* secretes prolactin and progesterone until the embryo has attached, and if not, a pseudo pregnancy will follow, lasting for 13 days. Pregnancy in the mouse lasts for 19-21 days (Hafez 1970).

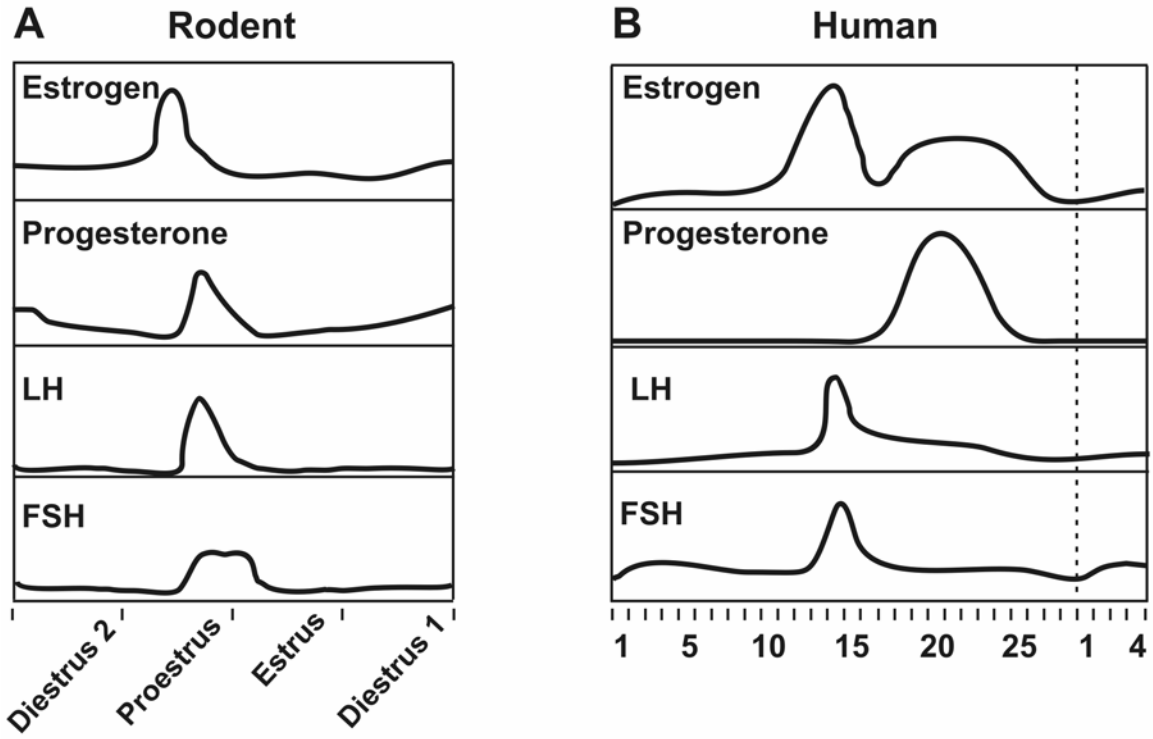

Figure 2. Comparison of the estrous cycle of the rodent (A) and the menstrual cycle in humans (B) (Adapted from Staley and Scharfman (2005)).

2.1.4. Peripheral estrogen effects

In addition to the impact on the ovarian cycle, estrogen and other sex hormones exert a wide range of other effects on the reproductive organs. In the uterine tube, estrogen promotes the progression of the egg cell whereas progesterone has opposite effects. Estrogen is also responsible for strengthening of the uterine mucosa after the ovarian cycle is over and it activates different glands to secrete, thus helping the egg cell to become attached should the fertilization have occurred. If the attachment of the embryo does take place, progesterone secretion increases, creating favorable conditions for the embryo. In general, estrogen affects the development of the reproductive organs already before puberty and promotes their maintenance after that time, as well as being involved in the secondary sex characteristics of females. The ovaries form an interactive hormonal regulation system with the pituitary gland and hypothalamus, such that the ovaries affect the functions of the other two sites via a so-called negative feedback mechanism. Thus, estrogen works in close interaction with other hormones. In addition to their effects on the reproductive organs, estrogens are critically important for the development of the mammary glands as well as for maintaining the shape and firmness of the skin in females. Also, estrogen has positive effects on liver metabolism and the maintenance of bone structure and function. Estrogen also influences the heart and other circulatory organs. Further, estrogen contributes to certain forms of cancer, e.g. breast cancer and certain cancers occurring in the reproductive organs. Additionally, estrogen has effects on the central nervous system (CNS) and thus, the brain.

2.2. Estrogen and brain

2.2.1. Estrogen receptors in the brain

Given the functional differences between ERα and ERβ, and the different contributions of separate brain areas to different functions, it would appear to be crucial to determine the spatial distribution of the ER subtypes in the brain in order to reveal the mechanisms behind estrogen-induced effects on brain functions. In general, it seems that both ER subtypes are rather similarly expressed throughout the brain, but in some brain areas one subtype is more dominant or even the only one expressed. The distribution of the two ER subtypes in both mouse and rat brain is summarized in Table 1. Only certain central brain areas related to cognitive functions are presented in the table and also the

ventromedial nucleus in the hypothalamus, a region important in the regulation of reproductive physiology and behavior. It can be observed that there are similarities but also differences in ER subtype distribution between the two rodent species, which also may be attributable to the different methodological approaches used in the studies.

	HC	Caudate putamen	VMN	Medial septum	Cortex
Mouse brain					
ERα	++	++	++	-	+
ERβ	+	+	-	+	++
Rat brain					
ERα	+	NA	++	+	+
ERβ	++	NA	-	+	+++

Table 1. Distribution of ERα and ERβ in the specific areas of the rodent brain. The data were collected from Mitra et al. (2003) for mouse brain (immunohistology) and from Shughrue et al. (1997) for rat brain (in situ hybridization). Abbereviations: HC, hippocampus; VMN, ventromedial nucleus of the hypothalamus; NA, not available. (+, small; ++, moderate; +++, high expression; -, no expression)

In addition to the genomic effects of estrogen in the brain, mediated through intracellular ERs, emerging evidence favors the existence of non-genomic effects and thus presumably membrane-related receptors may also occur in the brain. For example, within a time range of seconds to minutes, estrogen has been reported to induce rapid neuronal electrophysiological changes (Foy et al. 1999) and to activate several signaling cascades (Aronica et al. 1994, Singh et al. 1999). Interestingly, estrogen can affect transcriptional modulation by activating membrane-associated ER which in turn activates second messenger signaling pathways (Katzenellenbogen 1996). These effects are believed to be derived from modulation of intracellular signal transduction pathways, e.g. ERK/MAPK pathway (extracellular-signal-regulated kinase / mitogen-activated protein kinase) (Aronica et al. 1994, Singh et al. 1999). In addition to the circulating estrogen, as for many other steroid hormones, estrogen can be locally

synthesized in the brain (Stoffel-Wagner 2001, Zwain and Yen 1999). Aromatase, a crucial factor in local estrogen biosynthesis, is an enzyme that converts androgens to estrogens and which can be synthesized in neurons and astrocytes. It has been shown that adult hippocampal neurons synthesize estrogens *in vitro* and this synthesis is clearly attenuated by inhibition of aromatase activity (Prange-Kiel et al. 2003). Thus, the neurons in the CNS may receive estrogen either from its local biosynthesis from testosterone or from the periphery via the blood circulation.

2.2.2. Estrogen, neuroprotection and neuronal plasticity

The literature on estrogen's neuroprotective effects consists mainly from data of its antioxidant, defensive and neurone-sparing actions. Estrogen has been shown to have antioxidant effects against several free radical generators in many tissues and cell types (Behl et al. 2000). For example, estrogen can reduce β-amyloid (Aβ), haloperidol, and H_2O_2 -induced intracellular peroxides and attenuate the lipid peroxidation evoked by Aβ, glutamate toxicity or $FeSO_4$ exposure (Green et al. 2000). Perhaps the best known biochemical mechanism for the neuroprotective effect of estrogen is mediated via the MAPK-ERK1 pathway (Singh et al. 1999, Singer et al. 1999). It is likely that the MAPK pathway is not sufficient to account for all of estrogen's neuroprotective effects, though, since another candidate that might mediate estrogen's actions hand in hand with MAPK could be the Akt/protein kinase B pathway which acts by increasing the expression of an anti-apoptotic protein bcl-2, that in turn might be mediated via cAMP response element-binding protein (CREB) -dependent mechanisms (Pugazhenthi et al. 2000).

Possibly the most basic form of neuronal plasticity at the morphological level, in which estrogen has been shown to have positive effects, is the filopodial outgrowth, which occurs within minutes of estrogen exposure (Brinton 1994). These newly developed filopodia are an early sign of dendritic spines that will subsequently mature (Harris 1999). In their seminal paper, Woolley and McEwen (1994) observed that the number of dendritic spines in pyramidal neurons in the CA1 area of the hippocampus fluctuated according to the phase of the estrous cycle - and the amount of circulating estrogen and progesterone - in female rat. Namely, when estrogen levels are at their highest, the

number of dendritic spines also peak, while the progesterone levels seem to have an opposite relationship with dendritic spines. Further, the estrogen-induced effects proved to be dependent on glutamatergic N-methyl D-aspartate (NMDA) receptor activation. Not only did estrogen increase the amount of dendritic spines but it also elevated the number of synaptic connections (Woolley et al. 1997). Further proof for the hypothesis that estrogen-induced synaptic strengthening in the hippocampus is mediated via glutamatergic NMDA-dependent activation, was provided by an electrophysiological experiment, in which Rudick and Woolley (2001) observed that the NMDA-receptor, but not the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) -receptor, mediated sensitivity to synaptic input was increased by estrogen treatment. However, the estrogen-mediated increase in neuronal excitability is obviously not due to enhanced glutamatergic functions alone, since Murphy and Segal (1996) described estrogen's potency to modulate also the inhibitory GABAergic (gamma-aminobutyric acid) interneurons. Furthermore, estrogen treatments have been shown to increase neuronal excitability in various experimental models, such as hippocampal long-term potentiation (LTP) both *in vitro* (Bi et al. 2000, Foy et al. 1999) and *in vivo* (Cordoba Montoya and Carrer 1997) and hippocampal seizure susceptibility (Buterbaugh and Hudson 1991). Estrogen's ability to increase neuronal excitability has also clinical relevance. For example, some women suffer catamenial epilepsy, in which the seizure frequency or severity changes across the menstrual cycle. An increase in seizure severity has been observed at the mid-cycle when the ovarian hormones peak but also during the late luteal phase when the ovarian hormone levels start to decline (Herzog et al. 1997).

Modulation of neuronal excitability depends not only on estrogen's direct effects on cortical neurons but presumably also on its effects on different modulatory neurotransmitter systems, such as the cholinergic, serotonergic, noradrenergic and dopaminergic systems. In fact, estrogen's effects on the cholinergic system were among the first findings to indicate that gonadal hormones could possess actions outside their reproductive functions (Luine et al. 1980). The neurons of the cholinergic basal forebrain that project to cortex and hippocampus are believed to have a significant role in several cognitive functions. Experiments with rats have indicated that ovariectomy

(OVX) followed by short-term or long-term estrogen replacement therapy (ERT) increases the levels of choline acetyltransferase (ChAT), the rate limiting enzyme in acetylcholine formation, in the projection areas of the cholinergic basal forebrain (McEwen and Alves 1999, Singh et al. 1994). Moreover, the ChAT mRNA levels of the cholinergic basal forebrain seem to fluctuate during the rat estrous cycle (Gibbs and Aggarwal 1998). Possible mediators of the trophic effects of estrogen on cholinergic basal forebrain are nerve growth factor and brain-derived nerve growth factor (Gibbs and Pfaff 1992, Gibbs 2000b) which are produced in the hippocampus and retrogradely transported to basal forebrain cholinergic areas (Knipper et al. 1994).

Most of the serotonergic neurons in the brain are situated in or close to the Raphe nuclei in the brain stem (Jacobs and Azmitia 1992). The serotonergic system takes part in the regulation of many of the brain functions, including mood, aggression and cognitive processes (Bethea et al. 1998, Higley and Linnoila 1997, Rubinow et al. 1998). Increasing evidence, both from human and animal studies, suggests that estrogen has close interactions with the serotonergic system and thereby with the cerebral functions listed above. In view of the well known effects of serotonin on mood regulation, it is interesting that depression seems to be two to three times more prevalent in women than in men (Shively and Bethea 2004). Furthermore, ERT in peri- or postmenopausal women has been associated with improved mood and well-being, whereas ERT combined with progestin treatment has been reported to increase irritability and dysphoria (Genazzani et al. 2002). One possible mechanism to account for estrogen's effects on the serotonergic system could be inhibition of serotonergic neurons via 5HT1A autoreceptors (Bethea et al. 2002).

In addition to its actions on the cholinergic and serotonergic systems, estrogen also acts on catecholaminergic neurons. Noradrenergic system, emerging from the brain stem *locus coeruleus*, is known to have a role in vigilance, attention, cognitive processes and depression (Aston-Jones and Cohen 2005). Estrogen treatment has been shown to up-regulate both α- (McEwen 1980) and β-adrenergic (Vacas and Cardineli 1980) receptors in OVX female rats. Similarly to many of the effects of estrogen on the CNS, its effects on the noradrenergic system seem to depend on the balance between estrogen

and progesterone, since treatment with estrogen alone inhibits the synaptic reuptake of noradrenaline (NA) (Janowsky and Davis 1970) whereas estrogen combined with progesterone increases the reuptake (McEwen 1980). The dopaminergic system is known to be involved in executive functions and its disruption can lead to psychosis. In humans, estrogen can reduce the symptoms of tardive dyskinesia induced by L-dopa (Villeneuve et al. 1980). On the other hand, estrogen treatment in animal models has been reported to have different effects on the dopaminergic system depending on the brain area. For example, estrogen inhibits dopamine (DA) release in the median eminence (Cramer et al. 1979), but increases its release and turnover in the striatum (McEwen 1980) and downregulates striatal DA1 and DA2 receptors (Tonnaer et al. 1989). However, the mechanisms underlying the effects of estrogen on dopaminergic receptors are still largely unknown.

2.3. Estrogen and memory

In his book David Sweatt (2003) defined learning as acquisition of an altered behavioral response to a stimulus in the environment and memory as processes via which the learned information is stored. Recall, on the other hand, is understood as the event when this altered behavior is manifested. However, these definitions are not comprehensive, since learning may take place irrespective of whether any behavior related to the issue learned will ever be expressed or not. However, within the framework of this thesis these definitions are applicable as they permit an objective demonstration of learning in humans and animals.

Questions about the locus for learning and memory in the brain have undoubtedly challenged the imaginations of scientists for ages. The first answers to these questions appeared as late as the 1950's, when a patient called H.M., as well as some other similar cases, was reported to suffer of a profound amnesia following a bilateral resection of the hippocampus and adjacent structures in the medial temporal lobe (Scoville and Milner 1957). After the operation, H.M. was unable to form any new memories in normal every-day living. For example, he could not remember what he had had for breakfast or even that he had just eaten, nor could he remember the doctor he had spoken to five minutes previously. In contrast, he was able to communicate normally, and he was as

intelligent as before the operation. His memory for about three years before the operation had also partially disappeared, from that time he could only recall some trivial incidents. But his memory for the time before that time was well preserved. H.M. was able to learn motor skills, performing even very complex tasks. Thus, the observations from H.M. together with other unfortunate patient cases, led scientists to appreciate that there must be different neural systems serving different kinds of learning and memory: the hippocampus together with certain other medial temporal lobe structures were recognized as being crucial for forming declarative memories, although not being the site for the memory storage, and that there must also exist other neural systems contributing to procedural learning. The neural systems for both declarative and procedural memory in particular will be briefly described in the next chapters.

2.3.1. Different memory systems

Memory can be divided into two major classes: long-term memory (LTM) and short-term memory (STM) (see Figure 3 for the taxonomy tree of different memory systems). LTM is further divided mainly into declarative and procedural memory and also STM consists two sub-categories, sensory memory and working memory. Characteristics of LTM will be first described in more detail.

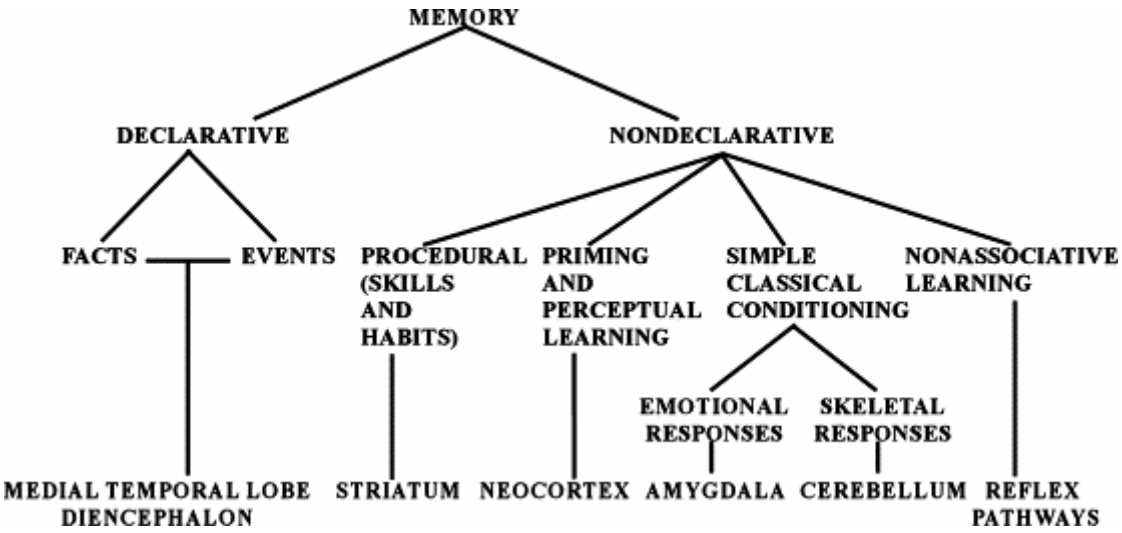

Figure 3. A taxonomy of the mammalian long-term memory systems. The taxonomy lists the brain structures thought to be especially important for each form of declarative and nondeclarative (procedural) memory. (Adapted from Squire (2004)).

2.3.1.1. LTM: Declarative memory

Declarative memory, also called explicit memory and which is further divided to episodic and semantic memory, is understood as a conscious memory for facts and events (Squire et al. 1993), requiring a "conscious" recollection, although this definition at least philosophically perhaps is not applicable for animals. Declarative memory is also fast but not always reliable, since a retrieval error might occur. It is a flexible memory, having access to several other memory systems (Squire et al. 1993). Thus, declarative memory is able to process and store the comparisons and relations between learning events and the items related to the learning events (Eichenbaum et al. 1992). Due to the multiple relations within the learned event, this representation is accessible in various different circumstances, also in situations different from the actual learning occasion, giving declarative memory its flexible nature (Eichenbaum et al. 1992).

As already mentioned, there are two types of declarative memory, episodic and semantic memory. Whereas episodic memory is a memory for personal events, having both spatial and temporal characteristics, semantic memory is a general knowledge about the world (Squire et al. 1993). For example, if I remember visiting my friend two days ago and eating there a steak and drinking wine, that is an episodic memory, but if I know that Helsinki is the capital city of Finland, that is a semantic memory. There is a connection between the two, though, since when creating semantic memories we often use our episodic memory. As in the case of H.M., amnesiacs with medial temporal lobe damage, have great difficulties in forming new episodic and semantic memories. Also, patients with damage mainly in their right frontotemporal regions experience difficulties in the retrieval of episodic memories (Kroll et al. 1997), while patients with damage in the corresponding area in the left hemisphere are unable to recollect their semantic memories (Markowitsch 1995).

2.3.1.2. LTM: Procedural memory

The properties and neural mechanisms for procedural memory markedly differ from those of declarative memory. Procedural memory (also called non-declarative or implicit memory) consists of four subtypes: skills and habits, priming, classical

conditioning and non-associative learning (habituation and sensitization). Procedural memory differs from declarative memory in the sense that it does not require conscious recall and also the learning process is more or less unconscious. It is also considered to be inflexible, since it can be used only in the same situation in which it was created, without having the relational and associative nature of declarative memory (Eichenbaum et al. 1992). Here it is reasonable to concentrate on procedural memory in terms of motor skills and habits, usually expressed through performance. For instance, once you have learned well how to sew, you can perform the task while you are watching television without having to concentrate all the time on the subtle motor movements of your fingers. The motor functions needed to accurately execute sewing, are not stored in muscles, however, but in the nervous system – and in a complicated way. This memory type utilizes certain brain areas such as cerebellum and basal ganglia. Also, patients suffering from Huntington's disease and Parkinson's disease which are characterized by severe pathology in the striatal areas, are reported to be impaired in tasks requiring procedural learning (Saint-Cyr et al. 1988). These observations underline the importance of the involvement of two different striatal nuclei, caudate nucleus and putamen, in procedural learning.

2.3.1.3. Working memory

Short-term memory is differentiated into two different categories, working memory and sensory memory. Whereas sensory memory is associated with implicit learning, working memory is an explicit memory, requiring conscious, active processes. According to the model proposed by Baddeley (2003), human working memory consists of three different systems, a central executive system and two systems for memory storage: the visuo-spatial sketchpad for storage of visual information and the phonological loop for verbal storage. The brain sites involved in these latter two processes have been proposed to be right and left temporoparietal lobes, respectively, and the frontal lobes for the central executive system (Baddeley 2003). In every-day life, working memory is needed for example in a situation you check a phone number in the book and "keep it in mind" for as long as you dial the number. For this short period of time the number is kept in your working memory but is lost from there within seconds after you have dialed the number.

2.3.1.4. Declarative memory in rodents

Why should one wish to study memory in rodents? The rationale for this question obviously is that useful animal models are needed to reveal the mechanisms of learning and memory and the results obtained can be extrapolated to humans e.g. in pathological states. However, when an animal model is used, one should bear in mind the special characteristics, especially those that are comparable to processes occuring in humans. Next the properties of rodent models of cognition will be briefly described and some attempts will be made to compare their analogies to humans.

The three main brain areas composing the declarative or hippocampal memory system are: 1) cerebral cortex, 2) the parahippocampal region, mediating connections between the cortex and hippocampus, and 3) the hippocampus (Eichenbaum 2000). In Fig. 4, these brain areas are visualized both in primates and rodents for comparison. The neural connections within this circuit are thought to function such that information, for example sensory information from corresponding sensory area or information from association areas, flows from the cerebral cortex to the two medial temporal lobe structures, wherein it is processed in two stages, modified and then sent back to the original site in the cortex. Eichenbaum (2002) proposed that the parahippocampal region is responsible for segregating the separate neuronal representations, of sensory or other modalities, transferred from cerebral cortex, and for keeping them in a "memory buffer" for a time frame of a few minutes, a gap between short- and long-term memory. During this gap, the hippocampus makes comparisons and relations between the given items and already established representations, then modulates them. Finally these representations are transferred back to the cerebral cortex via the parahippocampal region. These two processes together form the declarative memory. The difference between the functions of parahippocampal region and the hippocampus can be obtained from studies showing that the former is critical for certain types of recognition memory while the latter is essential for formation of memory of more complex relational aspects, contributing to the declarative memory.

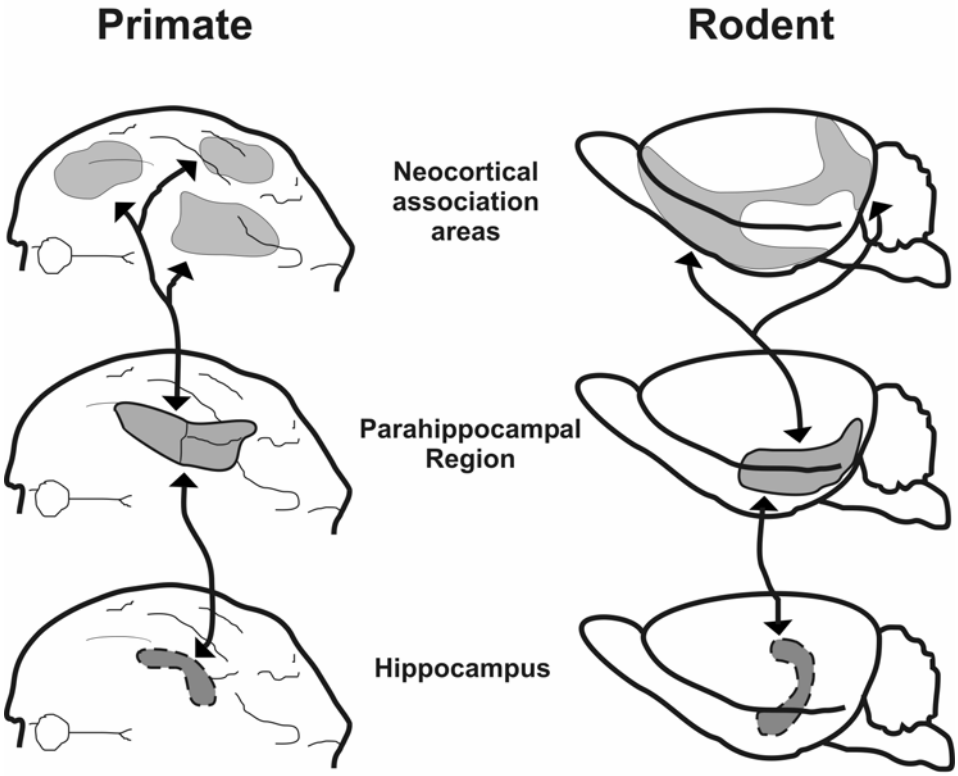

Figure 4. The anatomy of the hippocampal memory system. (Adapted from Eichenbaum (2000)).

Following the findings in patient cases like H.M. (Scoville and Milner 1957), who experienced severe amnesia after temporal lobe damage, the first attempts were made to create animal models of amnesia. In rodents, the most widely used method was to induce a lesion to the hippocampus combined with varying damage to the brain areas nearby. It was not until 1970's, however, that models showing clear impairment in learning and memory in rats with hippocampal lesion were successfully created. In 1978, O'Keefe and Nadel described their new findings, showing that in the rat hippocampus there are pyramidal cells, "place cells" that fire only at certain spatial locations of the environment (O'Keefe and Nadel 1978). They also found that rats with hippocampal lesions were impaired in certain tasks, e.g. those requiring spatial navigation, but not impaired in other tasks. These findings indicated that the hippocampus has a crucial role in the spatial performance and information processing. These findings proved that the hippocampus was able to form "cognitive maps" at least for spatial representations, differentiating its role from the processes involved in simple

habit learning. Thus, the hippocampus was determined to possess a role in declarative memory also in rodents.

In 1982 Richard Morris described the water maze test (the Morris water maze) that would become one of the most often used cognitive tasks in rodents (Morris et al. 1982). This task will be described in greater detail in Methods, but briefly, it consists of a circular pool filled with opaque water with an invisible platform just below the surface. The animals are given a number of trials and they are anticipated to find the platform by using extra maze spatial cues. Morris showed that intact animals learn the task quickly, improving their performance, i.e. finding the platform faster after each trial, whereas rats with a hippocampal lesion, although finding the platform somewhat faster in the later trials, still did not seem to remember the location of the platform, and they never learned to swim directly to the platform as the intact rats eventually did (Morris et al. 1982). This was confirmed in the subsequent "probe trial" during which the platform was removed. The intact animals kept on swimming around the location where the platform used to be, but the rats with hippocampal damage exhibited no preference for any quadrant of the pool. However, when a clearly visible platform, on which the animals could escape, was introduced both intact and lesioned animals performed equally well, swimming directly towards the platform and climbing on it, meaning that the impairment of the lesioned rats in the "spatial" version of the task was not dependent on visual or motor impairment, but selective incompetence for learning the spatial location of the platform.

Another widely used memory task, the radial arm maze, introduced by Olton et al. (1979), is somewhat different in its nature, but a sensitive task for detecting hippocampal dysfunction. The radial arm maze (RAM) is comprised of eight closable arms, radial to a central platform in which the animal can be confined between different trials. Depending on the version of RAM one, four or all eight of the arms are baited and the animal is expected to learn to collect each reward and avoid already visited arms by using extra maze spatial cues. Animals are able to remember the arms they have already visited within a single training session. Based on this finding, Olton defined working memory for rodents as memory allowing the animal to remember the arms it

had visited within a single session. According to Olton, working memory can be distinguished from reference memory which he claimed was a long-term association between different stimuli or a stimulus and response and acquired after repeated trials (Olton et al. 1979). The reference memory for RAM consists of aspects like there is food at the end of each arm or which of the arms has been baited etc. It is worth noting that Olton's definition for working memory, which is widely held among behavioral neuroscientists working with rodents, differs from the concept used in human psychology and primate studies. This mixed use of the term "working memory" has caused a great deal of confusion. One may argue that the memory of a rodent for the visited arms in RAM reflects an episodic encoding of the past events that the animal has to remember after variable intervals between the trials (Eichenbaum 2002). This is compatible with the studies showing only temporary impairment in this task after lesions of the prefrontal cortex, a pivotal brain area for working memory in monkeys, but severe impairment in the task in rats after hippocampal lesions (Becker et al. 1980).

Another task developed to study working memory in rodents is a delayed alternation in the T-maze task. Briefly, the animal is taught to collect a food reward in one of the arms in the T-maze so that after each trial the reward is switched to the opposite arm, i.e. the animal has to remember the arm it visited on the previous trial and enter to the other arm in the next trial to obtain the reward. Each testing session consists of several trials and the inter trial interval increases with the training sessions. Accordingly, to successfully complete the task, the animal needs to remember only the arm it visited on the previous trial and go to the opposite one. Therefore, until there is an inter-trial interval of some tens of seconds, this task requires a memory that shares the same characteristics as working memory in humans. Indeed, lesions in the prefrontal regions impair the rat's performance in this task (Brito et al. 1982). Compared to RAM, this task is more relevant to study working memory in rodents.

The third example of a behavioral test widely used to study declarative memory in rodents is the contextual fear conditioning task. In this task, the animal is placed in an operant box and thereafter a tone, lasting for several seconds, is present, followed by a mild foot shock through the floor. This procedure is repeated several times. After

several trials, the animal becomes conditioned to the tone, waiting for the aversive stimulus, the foot shock to appear. The animal's reaction is measured usually by certain behavioral responses characteristic for alert or fearful situation, such as freezing. Thus, the animal has learned that a tone will be followed by a foot shock. During the test trial, only the tone is presented, which alone causes a freezing behavior response. This response has been shown to be mediated by the amygdala and its connections with thalamus and cerebral cortex (Sweatt 2003). However, the trained rodent may freeze already when put into the operant box even when the tone has not been presented. On the other hand, if the animal is put in a different kind of test chamber it does not freeze until the conditioned stimulus i.e. the tone is presented. Thus, the behavioral response, freezing, depends on the context in which it has been learned on previous trials. It has been reported that hippocampal lesions abolish this behavior: the animal does not express the contextual fear conditioning (placement in the training chamber) but its conditioning to the tone is preserved (Sweatt 2003). Thus, the animal's capability of learning the context, i.e. the same location where the aversive stimulus was earlier experienced, is dependent on hippocampal function. This is an example where the rodent hippocampus is not only crucial for spatial learning, but also for other forms of declarative learning and memory.

2.3.1.5. Procedural memory in rodents

A classic test to study procedural habit learning in rodents is the position discrimination in the T-maze. The maze is a T-shaped apparatus, consisting of a stem arm and two side arms. Oliveira et al. (1997) explored the different learning mechanisms allowing rats to complete the task. The researchers used two different versions of the task, one having spatial cues and the other based on egocentric performance. In both versions of the task, rats with a hippocampal lesion and rats with a lesion in caudate-putamen were included. At the beginning of each trial, the animal was confined to the stem arm and then it could choose between the two side arms, left or right, but only the right arm was rewarded by a food pellet throughout the testing. Oliveira et al. (1997) noted that rats with hippocampal but not with caudate-putamen lesions were impaired in the spatial version of the task, whereas the latter lesion produced impairment in the egocentric version, the one requiring simple habit learning, where the rats with hippocampal lesions completed

the task as well as the control animals. This example illustrates the distinct contributions of the hippocampal and striatal functions on different maze learning strategies. It also underlines the importance of caudate-putamen area on stimulus-response based learning.

Further information of the role of the striatum on procedural learning has been gathered from tasks using operant conditioning. Operant conditioning is a learning paradigm in which a voluntary motor response is elicited to a given stimulus, thus differing from classical conditioning which is based on merely reflex or unconscious response to a stimulus. An example of an operant conditioning is a task in which an animal is taught to press down a lever during the presentation of a visual and/or acoustic signal, and is then required to hold the lever until a trigger stimulus occurs after an unpredictable delay in a time range of some seconds. The trigger stimulus informs the animal that it should now release the lever and press a second lever to obtain a food reward. The different subregions of striatum seem to contribute differently to performance in this kind of task, such that the dorsal striatum is involved in the correct utilization of external sensory information for the initiation of conditioned behavior, whereas the ventral striatum appears to be mainly concerned with the temporal expectation of the impending stimuli that trigger reward-reinforced movements (Florio et al. 1999). Furthermore, the dopaminergic innervation to the striatum is believed to play a crucial role in this kind of striatal learning (Florio et al. 1999).

The fear conditioning, described in the previous chapter, may be used to study multiple different memory systems. As described in the previous chapter, fear conditioning is a task used to assess the learning of association between the environment and a mild aversive stimulus. This task is relevant for use in rodent models of anxiety disorders. The animals are required to learn two facts: the training chamber refers to an aversive situation (the contextual conditioning) and that the conditioned stimulus, light or tone, predicts the coming foot shock (cued conditioning). The former response, as mentioned earlier, is dependent on hippocampal functions, whereas the cued conditioning is dependent on associative learning mediated by the amygdala (Sweatt 2003). Thus, the cued conditioning is a type of procedural, non-conscious learning.

2.3.2. Estrogen and memory in rodents

Perhaps the first indication of estrogen's capability in modulating cognitive functions was published in the late 70's by Luine and her coworkers in an experiment showing that estrogen treatment given to ovariectomized female rats affected the ChAT levels in the basal forebrain (Luine et al. 1980), a brain area later documented to have a role in cognitive functions and being also the site of extensive pathology in AD. Since that discovery, there has been much research done in this area and the effects of depletion and estrogen treatment have been studied in numerous studies in a wide variety of cognitive tasks in rodents. The experiments of this thesis were started in 1999 and after that time much new information has emerged in this field. For background, a brief summary of the reports on estrogen and memory in rodents appearing until the year 1999 is given here. Reports appearing after that time point are dealt with in the Discussion.

It was first documented that the density of dendritic spines in the CA1 area of the hippocampus varies during the female rat estrous cycle (Gould et al. 1990). This was followed by reports showing that fluctuations in gonadal hormones affect the sensitivity of the same area to LTP (Cordoba Montoya and Carrer 1997) and that estradiol treatment could enhance LTP (Wong and Moss 1992). Thus, it became of great interest to examine whether the gonadal hormones would have an effect also in learning and memory, especially in hippocampus-dependent processes. Most of the studies on gonadal hormones and memory in rodents have been conducted using rats and most often the cognitive tasks have been those measuring spatial learning. The attempts to find a correspondence between spatial learning and the aforementioned morphological and the functional changes occurring in the hippocampus across the estrous cycle have not yielded uniform results. Some studies have found a slight impairment (Galea et al. 1995, Warren and Juraska 1997) or no difference (Berry et al. 1997) in spatial water maze performance in rodents during proestrus, when the estradiol levels are high. Similarly, Markus and Zelevic (1997) showed impaired learning in a spatial-context conditioning task in proestrus rats. However, a delayed non-match to sample version of the radial arm maze found no difference in working memory errors between rats during

different stages of the estrous cycle (Stackman et al. 1997). In an interesting study by Korol and her co-workers (1996) reported that rats in proestrus preferably use a spatial, hippocampal strategy whereas rats in estrus, when the estrogen levels are low, prefer to use a response or striatal strategy in one version of plus-maze in which the animal may use different learning strategies.

Studies published before the 21st century on estrogen treatment, usually conducted in ovariectomized rodents, have also yielded somewhat inconsistent results. A study by Korol et al. (1994) showed impaired water maze performance in rats with estrogen treatment. On the other hand, some reports have found improved spatial learning in rats with a longer duration of estrogen treatment. For example, Daniel et al. (1997) found that ERT, administered via silastic capsules and started 30 days before the testing decreased working memory errors in RAM in OVX rats. Another report by Luine and her colleagues (1998) also showed that working memory but not reference memory errors were decreased after 12 days, but not after three days ERT in OVX rats. These reports do not support the hypothesis that the estrogen-induced increase in the number of CA1 dendritic spines (which is most reliably seen within 48 h after two daily estradiol injections) would be correlated with the changes observed in cognitive performance. Still, Packard and Heather (1997) found that a single estradiol injection given one hour after training and 24 h prior to testing did improve water maze performance. Dohanich et al. (1994) showed that in ovariectomized rats, single injections of estradiol alone (25 mg) or combined with progesterone (500 mg) reversed the scopolamine-induced impairment in T-maze alternation task. Also, acute but not chronic estrogen treatment has been shown to increase the dendritic spine density in the granule cell layer of the hippocampus, and accordingly, Henderson et al. (1996) found that acute but not chronic estrogen treatment improved radial arm maze performance in aged rats.

2.3.3. Human studies on estrogen and memory

There is some evidence that the phase of the menstrual cycle may affect cognitive processes also in humans. Most, although not all, studies exploring this question have found that women perform better in tasks requiring verbal and fine motor abilities when

they are at the midluteal phase of the cycle, i.e. when the levels of both estradiol and especially progesterone are high (Carlson et al. 2001, de Moraes et al. 2001, Fillenbaum et al. 2001, Matthews et al. 1999, Yaffe et al. 2000). These tasks are sexually dimorphic, meaning that women usually perform better in these tasks than men. Similarly, an interesting study by Maki et al. (2002) revealed that during the midluteal phase, young healthy women performed better in tasks measuring verbal and motor skills, compared to their performance during the follicular phase. Further, blood estradiol levels were associated positively with good performance in verbal tests, but negatively with tests on spatial abilities, that are also considered to be sexually dimorphic, but this time in favor of men. Therefore, it is probable that the estrogen levels, but not the progesterone levels, may be the key factor in mediating the changes in cognitive performance in different stages of the menstrual cycle.

As is also the case in rodents, studies on the effects of hormone replacement therapy (HRT) on cognitive performance in healthy postmenopausal women have yielded controversial results. A meta-analysis of 10 randomized controlled trials and eight cohort studies showed that healthy postmenopausal women suffering from postmenopausal symptoms who were given HRT had improved verbal memory, motor speed, vigilance and reasoning, whereas this effect was not seen in women receiving HRT but not suffering from postmenopausal symptoms (LeBlanc et al. 2001). The results of the large Women's Health Initiative (WHI) study indicated that HRT started after the age of 65 years seemed to have a slight deteriorating effect on cognitive abilities in postmenopausal women, especially in those already suffering of cognitive problems (Espeland et al. 2004).

2.4. Estrogen, aging and Alzheimer's disease

2.4.1. Menopause

Menopause is a period when the menstrual cycles become irregular and eventually end. It usually occurs around 45 to 55 years of age. The amount of the ovarian follicles decreases during a woman's reproductive life, and subsequently the levels of follicle stimulating hormone (FSH) increase (Lee et al. 1988). Subsequently, when the follicle cohort declines below a critical level, cycle changes start to occur. Levels of inhibin, a

peptide decreasing the secretion of FSH, decline as a result of the decreased follicle pool (Burger et al. 1998), further increasing the FSH content (Sherman and Korenman 1975). The next phase in the progress of menopause is characterized by anovulatory cycles accompanied by elevated estrogen production (Santoro et al. 1996). When three consecutive cycles have been missed, a woman has reached the late phase of menopausal transition (MT) (WHO Scientific Group 1996) and usually the very last menstrual cycle occurs within four years after this event (Taffe et al. 1997). The perimenopause is associated with certain symptoms, not directly related to ovarian function. Hot flushes are one of the typical symptoms at this phase (Santoro 2002). In the early MT, a woman may suffer from menstrual migraines (Lipton et al. 1999) and irritability which tends to occur though less frequently, in the late MT (Bromberger et al. 2001). Excessive or very minimal menstrual bleeding (Johannes and Crawford 1999), vaginal dryness and night sweats (Dennerstein et al. 2000), depression (Campbell and Whitehead 1977) and subjective experience of loss of general wellbeing (Santoro 2002) all belong to the symptomatology of MT.

In general, animals do not undergo menopause, the primates being the only exception. However, also in rodents, especially under laboratory conditions, similar physiological changes as seen in postmenopausal women can be observed and sometimes these animals are called estropausal. In the C57BL/6J strain of mice, the onset of the physiological state corresponding to menopause occurs around 13-15 months of age, when the mice become acyclic and experience lengthening of the cycle; the normally predominant 4-day cycles switch to predominantly 5-day cycles and later to even longer cycles (Felicio et al. 1984). The plasma estrogen levels are reduced and the preovulatory estrogen and progesterone pulses even become absent in these mice with lengthened cycles (Flurkey et al. 1982, Nelson et al. 1981). Experimental studies suggest that neuroendocrine changes play a crucial role in age-related changes of cyclicity and hormonal levels, although some of the changes are believed to originate from changes in ovarian functions. Neuroendocrine changes are thought to account for the major decrease of estrogen levels, whereas the aged ovary may be responsible for the decrease in the pre-ovulatory estrogen pulse (Felicio et al. 1986, Nelson et al. 1992).

Most often when using rodents, the postmenopausal state has been mimicked by ovariectomy (OVX), the removal of the ovaries, this being performed in either young or middle-aged females. This operation leads to a depletion of endogenous estrogen. Although ovariectomy is clearly not a perfect model of menopause, it has many advantages. First, when conducted on young or middle-aged rodents ovariectomy provides a tool to study the physiological responses of estrogen depletion alone, divorced from the aging process that naturally is related to perimenopausal age, and also to changes that estrogen depletion and aging might produce by interacting with each other. Another plus of this model is that it achieves a state that resembles the postmenopausal state. Namely, even at the age of 20 months, and even though no estrous cycles can be observed, the ovaries of an intact mouse might be functional to some extent and secrete low levels of estrogen, which is not the case in postmenopausal women. Furthermore, mice at the age of 20 months or older are also at a considerably high risk of experiencing a natural death, which complicates all experiments with aged animals. However, in order to have a rodent model of postmenopausal state which best mimics the situation in women, the animals should be aged, but even so, ovariectomy would be a rational experimental procedure to assure that the animals in use are at the same level in terms of their endogenous estrogen status.

2.4.2. Estrogen replacement therapy

Although naturally occurring, the postmenopausal state could be understood as a deficiency in the hormonal milieu and this deficiency has been tried to be restored with HRT, which consists of estrogen alone or combined with progesterone. Here the term ERT will be used whenever the treatment consists of estrogen alone and HRT if the treatment includes also progesterone. In industrial countries, peri- and postmenopausal women have used these therapies even in the absence of symptoms related to MT. The motives for the ERT or HRT have often been their hypothetical protective effects against cardiovascular diseases and osteoporosis. However, since chronic treatment with estrogen alone is known to increase the risk of uterine cancer, the prescriptions usually consist of HRT (Shapiro 2004). About 2/3 of menopausal women suffer from menopausal symptoms but only one of every three seek a medical cure for their symptoms (Prelevic et al. 2005). Typically these symptoms can be classified mainly in

two categories, vasomotor or psychosomatic, such as hot flushes and sweats. The symptoms are most pronounced during first few years after menopause, lasting for more than five years in about one fourth of women (Prelevic et al. 2005). ERT is reported to be effective against hot flushes in approximately 90 % of women (Shanafelt et al. 2002) and in general, subjects using HRT have reported of improved quality of life and well-being (Blumel et al. 2003).

Perhaps the most convincing and best documented positive effects that ERT produces on physiology of postmenopausal women are its effects against bone mass loss. ERT protects from bone mass loss, but only for as long as the therapy is continued and the reduced fracture risk induced by ERT is restored to the pre-ERT level five years after the treatment has been terminated (Delmas 2002). ERT's beneficial effects on bone mineralization in postmenopausal women seem to depend on the dose and modes of administration. In general, daily doses of 0.625 mg conjugated equine estradiol (CEE), 2 mg of 17β-estradiol or 2 mg of oral estradiol valerate and, in addition, the supraphysiological levels of estradiol seem to provide the optimal response (Lindsay et al. 1984, Selby and Peacock 1986).

Another suggested indication for ERT in postmenopausal women is its protective effect against cardiovascular diseases. Based on epidemiological studies especially from the early- and mid- 90's HRT was claimied to reduce the death rate and morbidity of coronary heart disease (CHD) by approximately 50 % in healthy postmenopausal women (Barrett-Connor 1991, Barrett-Connor and Bush 1991) and also in those already suffering from CHD (Henderson et al. 1991). Following these positive reports, HRT became one of the most common medications for postmenopausal women in industrial countries. However, these positive views for HRT have been challenged by more recent studies (Cherry et al. 2002, Clarke et al. 2002, Gami et al. 2003, Herrington et al. 2000, Hodis et al. 2003, Hulley et al. 1998, Viscoli et al. 2001) and in particular, the large clinical trials of WHI published in 2002 (Rossouw et al. 2002), which showed an increased risk of stroke, breast cancer, CHD and venous thromboembolism in postmenopausal women taking CEE combined with medroxyprogesterone acetate. Thus, long-term HRT probably cannot be recommended as a primary or secondary

prevention against CHD in postmenopausal women. The same is apparently true for stroke, since in addition to the WHI study (Rossouw et al. 2002) a few other recent studies have found an increased risk of stroke in women using HRT (Grady et al. 2002, Viscoli et al. 2001) and also a recent meta-analysis of several trials found a 29 % increase in the risk of stroke, especially ischemic stroke, in women using HRT (Bath and Gray 2005). Also, the large ERT study of WHI had to be stopped due to the increased risk of non-fatal stroke in women treated with estrogen alone (Anderson et al. 2004). In the mid 90's, some publications suggested that HRT might increase the risk of venous thromboembolism and pulmonary embolism (Daly et al. 1996, Grodstein et al. 1996, Jick et al. 1996) and these findings were confirmed later by the WHI study (Rossouw et al. 2002) reporting about two times higher risk of VTE in women using HRT.

ERT's impact on breast cancer has been widely debated. There is evidence that endogenous as well as exogenous estrogen affect the risk of breast cancer (Pike et al. 1993). A meta-analysis of several epidemiologic trials has shown that HRT indeed increases the risk for breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer 1997) and this generalization was further clarified by the WHI study (Rossouw et al. 2002). These harmful effects of HRT start to occur mainly after about four years since the start of the treatment. Interestingly, estrogen combined with progestin seems to pose a greater risk for breast cancer than estrogen treatment alone (Beral and Million Women Study Collaborators 2003, Li et al. 2003a). It seems that HRT has beneficial effects in some forms of cancer, however, since the WHI study (Rossouw et al. 2002) was the first to show that CEE+progesterone treatment leads to a decreased incidence of colon cancer and later another meta-analysis of 18 different studies confirmed this finding by detecting a decrease in colon and rectum cancers in HRT users (Gambacciani et al. 2003), with longer treatment proving to be more beneficial. This benefit occurred in the users of both estrogen alone or combined with a progestin (Newcomb and Storer 1995).

2.4.3. Epidemiologic studies on estrogen and Alzheimer's disease

Alzheimer's disease is an age-associated, neurodegenerative disease and the most common cause of dementias, usually occurring after 65 years of age. The incidence of AD increases after about 60 years of age, being 1 % among 60-64 years old and already about 40 % among individuals 85 years or older (von Strauss et al. 1999). The diagnosis of AD which is given after the findings of autopsy is based on the assessment of plaque frequency (Khachaturian 1985), neurofibrillary pathology stageing (Braak and Braak 1991) or both (Heyman et al. 1990). The neuropathological changes develop progressively, starting from the medial temporal lobes, first in the entorhinal cortex, hippocampus and subiculum. At later stages of the disease, the pathology extends to other cortical areas, especially association areas, but also to deeper structures of the brain such as the brain stem monoaminergic nuclei and cholinergic basal forebrain (Geula 1998, Price and Sisodia 1998).

Although a definite diagnosis of AD can not be given before affirmation of the AD-like neuropathological findings at autopsy, there are certain criteria for the diagnosis of probable AD based on clinical findings. The most prominent clinical feature of AD is a progressive loss of cognitive abilities. The cognitive decline typically begins by weakening of recent memory, whereas remote memories remain vivid. The memory deficit concerns selectively declarative memory, while procedural memory remains preserved (Forstl and Kurz 1999), presumably reflecting the severity of pathological features of AD, e.g. neuronal loss, especially in cortical and hippocampal areas (Cummings et al. 1998). Later on, as the disease progresses, the clinical symptoms include also language impairment, agnosias and apraxias and also visual and spatial deficits (Rossor et al. 1996), apparently reflecting the progression of the neuropathological changes to the associative cortex.

AD is more prevalent in women than in men, by about 1.6 fold, even taking into account the fact that women live longer than men (Gao et al. 1998). The gender difference seems to be most prominent among patients over 85 years of age (Fratiglioni et al. 2000). This fact has provoked researchers to speculate that reproductive hormones or the postmenopausal loss of hormones might be involved in this gender difference. In

the 90's altogether 12 case-control and cohort studies were conducted to investigate the possibilities of using ERT to prevent AD in postmenopausal women. In ten of these studies, women using ERT were found to have a significantly smaller risk of developing AD, compared to non-users (Sherwin 2002). Also, two meta-analyses, with somewhat different criteria and involving several observational studies, have found 29-34 % decreased risk of AD in the women using ERT compared to non-users (LeBlanc et al. 2001, Yaffe et al. 1998). However, recently published large randomized, double-blind, placebo-controlled trials of the WHI found that CEE combined with progestin seemed to even increase the risk of probable dementia and not to prevent mild cognitive impairment in women of 65 years or older (Shumaker et al. 2003). The results of the WHI study on estrogen treatment alone (CEE) proved to be very similar; ERT did not reduce the risk of dementia or mild cognitive impairment (Shumaker et al. 2004).

The effects of ERT on the clinical course of AD were also investigated extensively in 80's and 90's, but almost all of these studies suffer from several drawbacks in terms of selection criteria of the patients and other biases etc. More recently, more carefully executed clinical trials have been conducted. For example, two quite recent prospective, multicenter trials found no clinically beneficial effects of ERT on women with probable AD, neither with 12-month (Mulnard et al. 2000) nor with 4-month treatment (Henderson et al. 2000). Still, some studies have found positive effects with ERT in AD patients, e.g. on attention and verbal memory (Asthana et al. 1999) but in this particular study the sample size was a mere 12 patients, this same drawback being encountered in many of the published studies.

Whereas HRT was claimed to have preventive effects against AD, especially in the studies published at 80's and 90's, recent epidemiological trials have reported opposite effects. There are some fundamental differences between the studies that might account for their discrepant findings. First, in many of the studies conducted in the 80's and 90's, there are several confounding factors that might have skewed the results. Many studies have been based on population samples, not on placebo-controlled trials and therefore the study groups have contained natural biases for the use of HRT. For instance, usually women taking HRT were healthier and better educated compared to

non-users (Cauley et al. 1990) and these factors *per se* have been generally recognized as being protective factors against AD. Thus, the protective effects of HRT found in these studies might be biased by these issues. The recent negative findings of HRT on the incidence on AD (Shumaker et al. 2003, Shumaker et al. 2004) seemingly did not suffer from the problem of biased study groups, but instead these studies were conducted in women already 65 years of age or older, long after the perimenopause, a period of life commonly found to be most critical for initiation of HRT to gain its beneficial effects. In addition, in the WHI trials (Shumaker et al. 2003, Shumaker et al. 2004), the HRT consisted either of CEE alone or CEE combined with progesterone. CEE, which is a compound extracted from horse urine, contains several forms of estrogens and estradiol. CEE is a very common form of estrogen in the HRT provided in USA but, for example in Europe, CEE containing HRT preparations are very rare. In Europe, the estrogen used in HRT is mainly natural, synthetic estradiol. The difference between the different forms of estrogen preparations will need to be evaluated in future studies.

Studies examining the effects of ERT on cognition in AD patients have yielded mixed results. Several studies have reported that ERT improves cognitive performance, especially verbal memory in AD patients (Asthana et al. 1999, Henderson 1997, Resnick and Maki 2001), but some recent studies have not found any beneficial effect (Henderson et al. 2000, Mulnard et al. 2000, Thal et al. 2003, Wang et al. 2000).

2.4.4. Estrogen and the pathophysiology of Alzheimer's disease

The etiological background of AD remains unknown (Smith 1998). The most widely accepted theory about AD pathophysiology is the so called Aβ cascade hypothesis (Selkoe 2003). This is based on the findings that all known mutations linked with familial, early-onset AD, including amyloid precursor protein (APP), presenilin 1 or 2 (PS1, PS2) mutation, increase the production or aggregation of Aβ (Selkoe 1997).

Aβ is derived from its precursor protein APP by proteolysis and is expressed in very small quantities under normal physiological conditions (Haass et al. 1992), but an abnormal APP metabolism will lead to increased production of Aβ, a phenomenon

characteristic of AD pathology (Selkoe 1997). APP is a transmembrane domain protein, expressed throughout the body (Selkoe 1999), having three different isoforms of 695, 751 and 770 residues in humans, the first of these being the most common form expressed in the brain. APP can be metabolized along two different pathways, either via the α-secretase pathway or the β-secretase pathway (Hardy 1997). These two pathways are illustrated in Fig 5. The deposition of Aβ42 is thought to be an early sign of AD, followed later by the deposition of Aβ40 (Jarrett et al. 1993). The neurotoxicity of aggregated Aβ is based on its ability to induce activation of the microglia and astrocytes surrounding the Aβ depositions causing them to release cytokines and acute-phase proteins (McGeer and McGeer 1995) leading to an inflammatory responses in the neurons nearby. Other neurotoxic effects of Aβ have also been reported, such as disruption of calcium homeostasis (Joseph and Han 1992) and oxidative damage (Thomas et al. 1996). It has been suggested that Aβ accumulation might also accelerate or induce tau phosphorylation and subsequently the formation of neurofibrillary tangles (Selkoe 2001). In summary, these effects may lead to observed synaptic and neuronal loss observed in AD (Selkoe 2001).

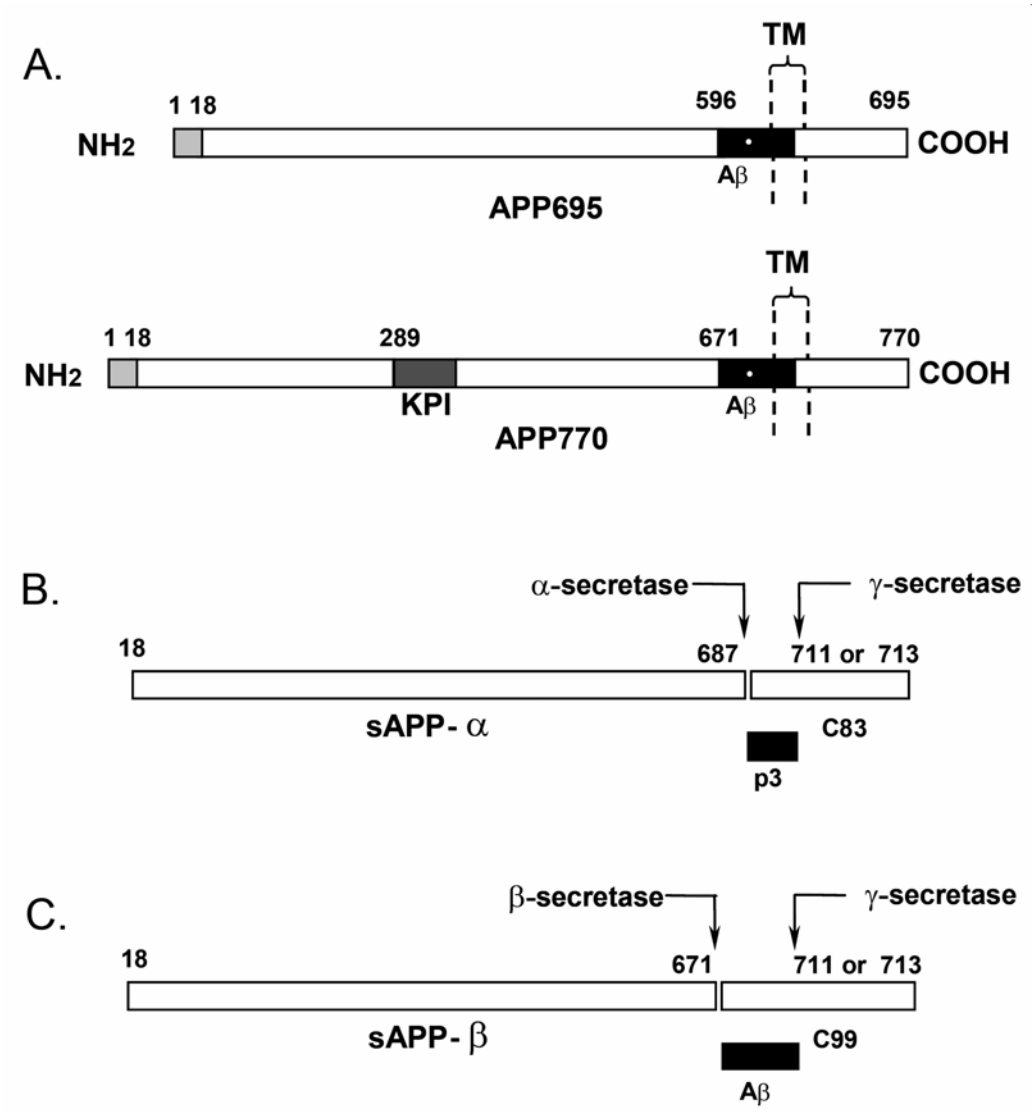

Figure 5. Schematic diagrams the APP protein with the two pathways that process APP. (A). APP has a single membrane-spanning domain (TM), and a 17-residue signal peptide at the N terminus. The diagram illustrates two APP alternate splice forms, APP695 and APP770. (B) α-secretase cleaves APP after residue 687, resulting in the secretion of the soluble ectodomain of APP (sAPP-α) into the medium and retention of carboxy-terminal fragment C83 in the membrane. The C83 fragment can undergo cleavage by γ-secretase at residue 711 or residue 713 to release the p3 peptides. (C) Alternatively, β-secretase can cleave APP after residue 671, resulting in the secretion of the slightly truncated sAPP-β, and the retention of C99, which can be further cleaved by γ-secretase at residue 711 or residue 713 to release the Aβ40 or Aβ42 peptides. (Adapted from Selkoe (1999)).

One of the neurotransmitter systems most profoundly affected in AD is the cholinergic system. Loss of cholinergic neurons in the basal forebrain and decreased acetylcholine (ACh) levels are characteristic findings in AD brains (Whitehouse et al. 1982). As already mentioned, OVX decreases and estrogen increases the amount of ChAT in the rodent brain (Luine et al. 1975). Thus, some of the proposed benefits of estrogen on decreasing the incidence or alleviating the symptoms of AD have been claimed to be attributable to its effects on cholinergic function. Interestingly, in a post-mortem study examining the brains of AD patients, Ishunina and Swaab (2001) found an upregulation of ERα in nucleus basalis of Meynert, a crucial brain area in human brain cholinergic system.

Another pathological feature of AD which can be modulated by estrogen, is the Aβ accumulation. In cell culture models, estrogen has been shown to promote the production of the non-amyloidogenic form of APP (sAPP) and to reduce the production of Aβ (Jaffe et al. 1994, Xu et al. 1998). Furthermore, even a 50 % increase in Aβ levels induced by OVX in guinea pigs, has been partially restored by treatment with estrogen for 10 days (Petanceska et al. 2000). In a double transgenic mouse line, i.e. mice with mutations in both APP and PS1 and having elevated brain Aβ levels, OVX has been found to elevate Aβ levels 3-4 months after the operation (Zheng et al. 2002) and a 50 % increase was found in Aβ levels in another APP transgenic mice 5 months after OVX (Levin-Allerhand and Smith 2002) whereas OVX lasting for 6-8 weeks had no effects on brain Aβ levels in the mice of the same strain (Levin-Allerhand et al. 2002). Estrogen treatment, on the other hand, has had more uniform effects on Aβ levels. In the study by Zheng et al. (2002) the increased Aβ levels in brain of OVX mice were partially or fully reversed and similar results were obtained in the study of Levin-Allerand et al. (2002). Thus, these results indicate that the failure in endogenous estrogen production might lead increased levels of brain Aβ and thus to an increased risk of developing AD, whereas the estrogen treatment might have a protective effect under these conditions.

In addition to estrogen's abilities on modulating Aβ metabolism and the cholinergic system, estrogen has been shown to have effects on other characteristics of AD pathology, such as oxidative stress and other neuroprotective effects, and these effects have been briefly described above (see chapter 2.2.2.).

3. AIMS OF THE STUDY

Alzheimer's disease is more common in women than in men, and it has been proposed that the decreasing levels of estrogen in postmenopausal women might have a role in this phenomenon. Further, it has been suggested that estrogen treatment might have beneficial effects on cognitive performance in postmenopausal women and also that it might be able to delay the onset of AD or ameliorate its symptoms.

We wanted to investigate these properties in an experimental mouse model of AD and to shed light on some effects of estrogen on cognitive performance.

The specific aims of this study were:
I) To examine the cognitive effects of long-term, tonic estrogen treatment on two cognitive tasks, utilizing two different kinds of memory in adult, ovariectomized mice. Also, we wanted to determine the effects of estrogen treatment on the levels of hippocampal neurotransmitters.

II) To evaluate whether different modes of estrogen treatment, i.e. tonic vs. phasic treatment, would lead to different outcomes in the same tasks as in (I) and whether changes in the levels of hippocampal aromatase (CYP 19), ERα and ERβ expression would correlate with the cognitive performance.

III) To test whether aging and long-term estrogen depletion could attenuate the beneficial effect of estrogen on memory.

IV) To evaluate the effects of long-term tonic estrogen treatment on cognitive performance and amyloid beta accumulation in female mice carrying mutated human APP and PS1 genes.

4. MATERIALS AND METHODS

4.1. Animals

The strain, gender and age of the mice used in these experiments were as follows:
Study I: female C57BL/6J (n = 95, weight 20-40g, 7 months of age, Kuopio, Finland) and male C57BL/6J mice (n = 26, weight 30-40g, 10 months of age, Kuopio, Finland).
Study II: female C57BL/6J mice (n = 82, weight 20-40g, 5 or 11 months of age, Kuopio, Finland)
Study III: female C57BL/6J mice (n = 112, weight 22-36g, 7, 11 or 24 months of age, Kuopio, Finland)
Study IV: female APPswe and PS1(A246E) double transgenic mice (AP mice) and wild-type C57BL/6J littermates (n = 225, weight 20-40g, 6, 9, 12 or 17 months of age, Kuopio, Finland)

The double transgenic AP mice were generated from matings between APPswe and PS1(A246E) transgenic mice that were generated by Borchelt et al. (1997) at Johns Hopkins University (Baltimore, MD, USA) and are now bred locally in the National Laboratory Animal Center of the University of Kuopio.

The mice were individually housed in a controlled environment (temperature 21°C, humidity 50-60%, lights: 7:00 –19:00). Food was available *ad libitum*, except during behavioral testing in the radial arm maze and T-maze, when the mice were food deprived to 85–90% of their free feeding body weight. Water was freely available at all the times. The studies were conducted according to guidelines set by the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland.

4.2. Procedures for surgical operations and estrogen treatment

The ovariectomy was conducted under anesthesia (pentobarbital + chloral hydrate (50/50); 36 mg/kg, i.p.). An incision was made in the back and the ovaries were removed and the muscles and skin were stitched. The sham animals were given only the incision on the skin, but the ovaries were not touched. The estrogen treatment was conducted using minipellets containing 0.18 mg of 17β-estradiol (Innovative Research of America, Sarasota, FL, USA), releasing estradiol for 90 days (I-IV) or via daily i.p.

injections of 17β-estradiol (20μg), diluted in sesame oil (II). The pellets were implanted s.c. in the upper neck under anesthesia (pentobarbital + chloral hydrate (50/50); 36 mg/kg, i.p.). The non-treated control animals were given only an incision in the skin. After each experiment the mice were sacrificed by cervical dislocation and the uterine weights were measured for female mice.

4.3. Behavioral testing

4.3.1. Radial arm maze (I-IV)

The 8-arm radial arm maze (RAM) was located in a dimly lit room with a rich environment of extra-maze cues. The RAM (Crusio et al. 1987) had a central platform (diameter 20 cm) and 8 arms (30 cm x 6 cm), which all have walls (6 cm high) made of transparent plexiglas. The 8 arms were separated from the central platform with guillotine doors. A 2 cm high opaque plastic sheet located 7 cm from the distal end of each arm prevented the mouse from seeing the food reward. To prevent the use of olfaction as a guide to the baited arm, the stock of rice cereal used as rewards was placed behind a perforated wall 3.5 cm from the distal end of the arm. The mice were first familiarized with the RAM by letting them explore it freely, all arms open for 10 min on two days, during which time the food reward was available at the end of each arm. The task itself was a simplified version of the Jarrard's (1978) 4 baited, 4 non-baited arms RAM task. The RAM version used in this study (1 baited, 7 non-baited arms) makes a distinction between reference memory errors as initial visits to non-baited arms and working memory errors as subsequent visits to non-baited arms during the same trial. After familiarization, the mice were trained to enter one baited arm of the maze and to avoid the remaining seven non-baited arms. The baited arm remained the same throughout the testing. Each trial began with the placement of the mouse on the center platform with all doors closed. After 5 s, all doors were opened. Each trial was completed when the mouse reached the end of the baited arm and returned to the center platform after consuming the food reward. A 30 s intertrial interval was introduced before the start of the next trial. The mice were given eight daily trials (each consisting of the total number of arm visits required until the mouse found the food reward) and trained for five days. The number of reference and working memory errors were recorded separately. Earlier findings in our laboratory indicate that mice first learn to avoid re-entering the same arms, and only slowly learn to prefer the baited arm.

Furthermore, in this task, dorsal hippocampal lesions in mice increase the number of working memory errors but not reference memory errors (Rissanen 1999).

4.3.2. T-maze (I-IV)

The T-maze consisted of a stem (38 cm x 7 cm) and two arms (35 cm x 7 cm). A sliding door separated the first 11 cm of the stem as the starting compartment, and a door at the each arm separated the arm from the stem 8.5 cm from the intersection. The walls were painted black and were 14 cm high to encourage the use of an egocentric response strategy. The source of illumination was an incandescent light located above the stem of the maze. The mice were first familiarized to the T-maze for two days by letting them freely explore it for 10 min until they repeatedly located and ate the rewards (rice cereals) at both arms of the maze. After two days of pretraining, testing was conducted as follows. In the initial trial of the first testing day, both arms of the maze were rewarded to test the spontaneous turning preference of the animal. After this trial, the arm that the mouse had not spontaneously selected was rewarded for 15 consecutive trials. A 1-min intertrial interval was introduced between the trials during which time the animal was confined in the starting compartment. At the beginning of each trial, the animal was given 5 s to leave the starting compartment. If this did not happen, the mouse was encouraged to start by gently pushing it with a paintbrush (encouraged start). The rewarded arm remained the same for all three days of testing. The following parameters were recorded: the number of correct choices and the number of trials that the mouse had to be encouraged to start. Previous studies in our lab indicate that a fimbria-fornix lesion does not affect performance in this task, so we use it as a control memory task that should be independent of hippocampal functioning (Liu et al. 2002).

4.3.3. Morris water maze (IV)

We used a black plastic circular pool, diameter 120 cm, and a black painted stainless steel square platform; 14 x 14 cm, 1.0 cm below the water line. The platform was located in one of the four quadrants halfway between the wall and pool center. The starting locations, which were labeled North, South, East and West, were located arbitrarily on the pool rim. The timing of the latency to find the submerged platform was started and ended by the experimenter. A computer connected to an image analyzer (HVS Image, Hampton, UK) monitored the swim pattern. Mice were placed in the water with their noses pointing towards the wall at one of the starting points in a

random manner. If the mouse failed to find the platform within the maximum time, it was placed there by the experimenter. Mice were allowed to stay on the platform for 10 s. A 30 s recovery period was allowed between the training trials. The temperature of the water was kept constant throughout the experiment (20.0 ± 0.5 °C). The training schedule consisted of 7 consecutive days of testing. During the two first days of testing, the mice were trained with a visible platform for five 50 s trials per day. During visible platform training, the platform location was changed after each trial. During the 5 following days of testing, the mice were trained with the hidden platform for five 50 s trials per day. The platform location was kept constant during this period of training. After the fifth trial on the seventh day, the platform was removed and the mice were allowed to swim for 50 s. During the platform training trials, the swim speed, path length and latency to find the platform were measured. Because of the significant treatment effect on swim speed, only path length was used in the statistical analyses. During the spatial probe trial, the time spent in the target quadrant in which the platform had previously been located was measured. The spatial version of the task is sensitive for detecting hippocampal learning whereas the cued version is used as a control task, detecting possible motor or visual dysfunctions.

4.4. Other measurements

4.4.1. Neurochemistry (I)

After all behavioral testing, the mice were sacrificed by cervical dislocation and the body and uterus weights were measured. The brains were removed and dissected on ice for later neurochemical measurements. The hippocampal noradrenaline (NA), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), and dihydroxyphenylacetic acid (DOPAC) levels were determined using high-performance liquid chromatography (HPLC) with electrochemical detection as previously described (Jakala et al. 1992). The hippocampal ChAT activity levels were measured using a radiochemical method as described earlier (Fonnum 1975).

4.4.2. Expression analyses

The reverse transcription polymerase procedure was performed using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems) to generate cDNA in a 25 µl reaction volume from 0.5 µg of total RNA. Relative quantifications of mouse CYP19, ERα and ERβ were calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as

the endogenous control. Real-time quantification of CYP19, ERα and ERβ gene expressions were carried out using ABI PRISM® 7000 Sequence Detection System with FAM dye-labeled fluorescent assays (Assays-On-Demand™ Gene Expression Products, Applied Biosystems), and the endogenous control gene, GAPDH with VIC dye-labeled TaqMan® Rodent GAPDH Control Reagents kit (Applied Biosystems). Amplifications were performed in singleplex reactions with 10 to 40 ng cDNA in a 15 µl reaction volume with PCR cycling from 40 to 44 cycles. The amplification of GAPDH was performed with 50 nM GAPDH primers and 200 nM GAPDH probe.

4.4.3. Serum estradiol levels (II)

For the measurement of serum estradiol levels, the blood samples were collected from the femoral vein and finally by cardiac puncture. The serum was separated by centrifugation and stored at –20°C until estradiol measurement. The serum estradiol concentrations were measured using commercial RIA kits (Wallac Delfia; Perkin Elmer, Turku, Finland) as described in Rulli et al. (2002).

4.4.4. Aβ40 and Aβ42 ELISAs (IV)

The hippocampi were homogenized in guanidine buffer (5.0 M guanidine-HCl/50 mM Tris-HCl, pH 8.0) in proportion to their weight. The samples and Aβ-peptides used as standards were prepared to contain 0.5 M guanidine-0.5% BSA-1 mM AEBSF in the final composition. The levels of Aβ40 and Aβ42 were quantified using the Signal Select™ Beta Amyloid ELISA Kits for human Aβ (BioSource International Inc.) according to the manufacturer's protocol. The Aβ40 and Aβ42 levels were standardized to brain tissue weight and expressed as ng (Aβ)/g (brain tissue).

4.5. Experimental design

Study I

10-month-old male (n = 26) and 7-month-old female (n = 95) C57BL/6J mice were used in this study. The female mice were sham-operated (SHAM) or ovariectomized (OVX) at the age of 3 months. Two groups from both SHAM and OVX mice were treated with subcutaneous estrogen pellets, which were implanted 7 days (SHAM/OVX + 7 days E) or 40 days (SHAM/OVX + 40 days E) before behavioral testing was started at the age of 7 months. One group of male mice received estrogen pellets 7 days before behavioral

testing at the age of 10 months, while the control male group was left intact. The mice were tested in RAM and the position discrimination task in the T-maze. After the behavioral tasks were completed, the mice were sacrificed and the hippocampi were removed for the measurement of the monoamines.

Study II

This study consists of two experiments, referred to as Experiment I and Experiment II. In both experiments the mice were ovariectomized (OVX) at the age of three months. The OVX mice received estrogen treatment for the last five weeks of their lifetime and the control groups of OVX mice were given a placebo treatment. The estrogen was administered either via daily injections (i.p., one hour prior to behavioural testing) (n = 11) (Experiment I) or via subcutaneously implanted estrogen pellets (n = 10) (Experiment II). The mice were tested in RAM and the position discrimination task in the T-maze. The mice were killed at the age of 4.5 months in Experiment I and at the age.of 11 months in Experiment II. The serum estradiol levels were measured using the DELFIA kit in an additional group of OVX mice with similar estrogen treatments as in Experiment I and II. For reverse transcription polymerase chain reaction (RT-PCR) analysis, the brain was removed and hippocampi were dissected. Brain samples were frozen in liquid nitrogen and then stored in -70ºC until RNA extraction.

Study III

Female (n = 112; 7, 11 or 24 months of age when tested) C57BL/6J mice were used in this study. This study consisted of two experiments, Experiment I and Experiment II. In Experiment I, the mice (n = 38) were either OVX or sham-operated (SHAM) at the age of 5 months. At the age of 23 months, i.e. 18 months after the operation, half of the OVX (OVX+E, n = 14) and SHAM (SHAM+E, n = 6) mice were treated with estradiol containing minipellets, while the other half was left untreated (OVX-group, n = 11; SHAM-group, n = 7). Treatment with estrogen pellets was started 40 days before the behavioral testing and continued throughout the testing. The animals were tested in RAM and T-maze at age of 24 months. The effects of estrogen treatment on maze learning in 24-month-old OVX mice in this study were compared to the earlier results Study I.

Experiment II consisted of only SHAM and OVX mice tested at different ages (7, 11 and 24 months of age) and concomitantly after different OVX durations (4, 8 and 19 months, respectively): 7-month-old mice from Study I, (SHAM: n = 28, OVX: n = 14, OVX duration 4 months), 11-month-old mice (SHAM: n = 16, OVX: n = 16, OVX duration 8 months) and 24-month-old mice from Experiment I (SHAM: n = 7, OVX: n = 11, OVX duration 19 months). The performance of the mice in RAM was compared in Experiment II.

Study IV

Four age groups (3-6, 6-9, 9-12, and 14-17 months of age) AP and control mice were used. Behavioral analyses were performed for the three youngest age groups (3-12 months), histological analyses for two age groups (9-12 and 14-17 months) and biochemical analyses for all four age groups. All the control mice were sham-operated (SHAM), whereas the AP mice were either sham-operated or ovariectomized (OVX) at the age of 3 months. In the behaviorally tested age groups, half of the AP OVX mice (AP OVX+E) and in the oldest group (14-17 months), half of the AP SHAM mice (AP SHAM+E) received estrogen treatment for the last 3 months of their lifetime. Accordingly, the following treatment groups were used: 3-12 months old mice: control SHAM, AP SHAM, AP OVX and AP OVX+E; 14-17 months old mice: control SHAM, AP SHAM, AP SHAM+E and AP OVX. The estrogen was administered via subcutaneously implanted estrogen pellets. The behavioral testing in T-maze, water maze and RAM was started two months after the initiation of estrogen treatment in AP OVX mice and one month before the mice were killed at the age of 6, 9 or 12 months. The AP mice were used for behavior (n = 167), for brain Aβ biochemistry (n = 143) and/or histology (n = 75). For biochemical measurements, the A/P mice were killed by cervical dislocation, the brain was removed and the hippocampi were dissected. Brain samples were then frozen in liquid nitrogen and stored at -70ºC until hippocampal Aβ40 and Aβ42 levels were determined using ELISA. The histological amyloid plaque measurements were performed on AP mice at the ages of 9 (age of appearance of first amyloid plaques) and 17 (age of abundant amyloid deposition) months. At the age of 9 months, the mice (n = 25) were transcardially perfused. At the age of 17 months (n = 50), the mice were killed by cervical dislocation, and one hemibrain was immersed in paraformaldehyde, while the other hemibrain was dissected to frozen samples.

4.6. Statistical analyses

All statistical analyses were carried out using SPSS for Windows software (versions 8.0 - 11.5, SPSS Inc., USA). The effects of mouse age, surgery, estrogen treatment, mouse genotype and their interactions with behavioral measures (RAM, T-maze and water maze) were analyzed using univariate analysis of variance (ANOVA) or General Linear Model for repeated measures (rmANOVA). The neurochemical variables, body and uterus weights and Aβ levels were analyzed with t-test or ANOVA. The correlations between behavioral measures and neurochemical variables, ER contents or Aβ levels were analyzed with either Pearson's or Spearman's correlation analysis.

5. RESULTS

5.1. Effects of ovariectomy and estrogen treatment on maze learning and hippocampal neurotransmitters

5.1.1. Findings in the memory tasks

In RAM, the female mice improved their performance during training days as measured by the decrease in the number of reference and working memory. The numbers of working and reference memory errors were higher in OVX than in SHAM (I, Fig. 1B,E). The rmANOVA also revealed an overall estrogen treatment effect on both reference and working memory errors and there was an operation by treatment interaction in working memory errors (I, Fig. 1B,E). A further comparison of working memory errors individually in the OVX and SHAM groups revealed a significant difference between treatment groups among OVX mice and a marginally significant treatment effect in SHAM (I, Fig. 1). The subsequent contrast analysis indicated that in the OVX group, the mice receiving 7 days of estrogen treatment made less working memory errors than non-treated mice, and the mice given 40 days of estrogen treatment made less errors than non-treated mice and mice with 7 days of treatment. The male mice improved their performance over the training days as well, as measured in a decrease in the number of reference and working memory errors. The rmANOVA also revealed an overall estrogen treatment effect and day by treatment interaction in working memory errors. The estrogen main effect on working memory errors was largely due to a group difference on the first training day, which was confirmed by t-test on the individual training days (I, Fig. 1F).

In the T-maze, the female mice improved their performance over the training days as measured by an increase in the number of correct choices. The rmANOVA showed an overall estrogen treatment effect and operation by treatment interaction. An individual analysis of the OVX and SHAM groups revealed a significant difference between the treatment groups in the number of correct choices among OVX mice (I, Fig. 2A,B). The subsequent contrast analysis indicated that in the OVX mice both the short- (7 days) and long-term (40 days) estrogen treatments increased the number of correct choices compared to no treatment. The ANOVA revealed an overall estrogen treatment effect on the number of encouraged starts and an operation by treatment interaction. An individual analysis of the OVX and SHAM groups revealed a significant difference

between treatment groups in encouraged starts among OVX mice. The numbers of encouraged starts for different groups are displayed in I, page 27. The male mice improved their performance over the training days as measured by an increase in the correct choices. The analysis also revealed a day by treatment interaction in correct choices (I, Fig. 2C), which was due to a different slope of the learning curves of the treatment groups. There was no difference in the number of encouraged starts between treatment groups.

5.1.2. Neurochemistry

The effects of operation, treatment and their interaction on hippocampal neurochemistry in female mice are summarized in Table 1 (I). In the male mice, there was no difference in the hippocampal ChAT activity between estrogen-treated and non-treated male mice. When the neurochemical parameters were used as covariates in the rmANOVA of T-maze and RAM data, no significant effects for covariates were found (data not shown).

5.2. Effects of estradiol on spatial learning, hippocampal aromatase, and estrogen alpha and beta mRNA levels

5.2.1. Findings in the memory tasks

The animals improved their performance in RAM during the testing days as measured by the decreased number of both working memory errors and reference memory in both Experiment I and Experiment II. There were no differences between the groups in working memory errors either in Experiment I (Fig. 6A) or Experiment II (Fig. 6D). With respect to reference memory errors, there were no differences between the groups in Experiment I (Fig. 6B), but in Experiment II the mice receiving tonic ERT outperformed the OVX mice (Fig. 6E).

The animals improved their performance in the T-maze during the testing days as measured by the decreased number of errors in Experiment II, whereas in Experiment I no improved performance could be detected. In Experiment I, there was a non-significant trend toward better performance in OVX mice compared with mice treated with phasic ERT (Fig. 6C), whereas in Experiment II there were no differences between the groups (Fig. 6F).

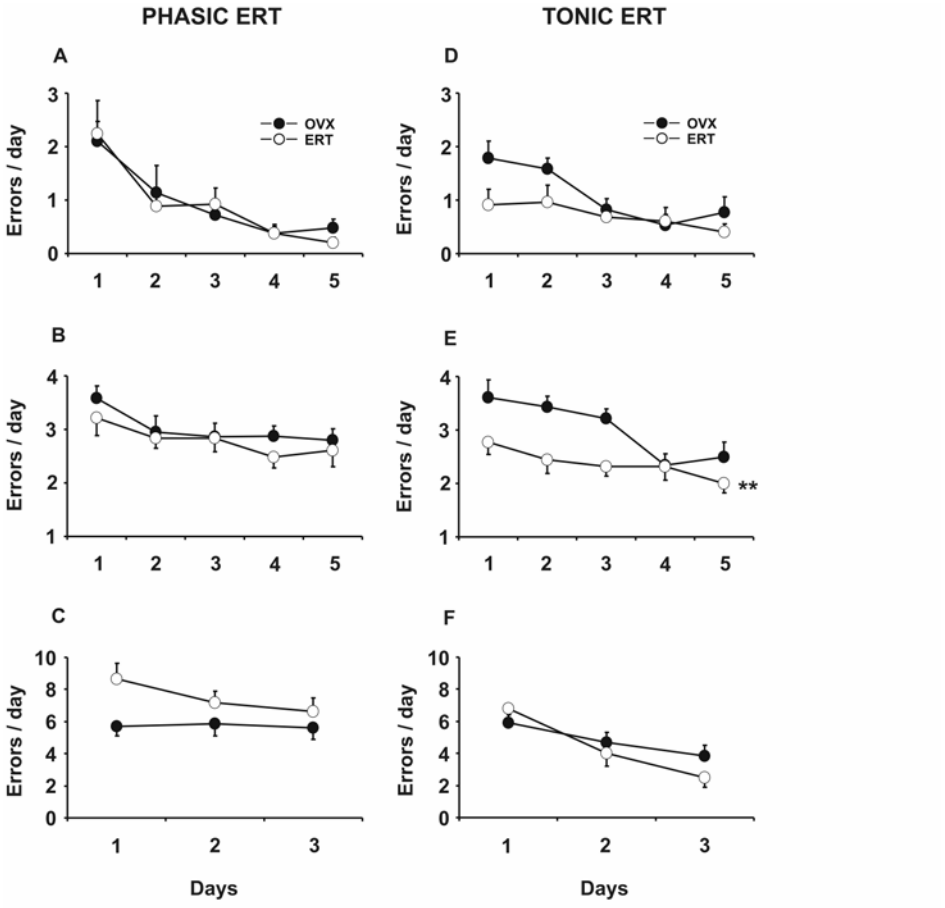

Figure 6. Effects of ovariectomy (OVX) and estrogen treatment on RAM and T-maze performance. Lines represent means of daily number of errors and ± SEMs over five testing days in RAM (working memory errors: AD; reference memory errors: BE) and over three testing days in T-maze (T-maze errors: CF); ** < 0.01.

5.2.2. CYP19, ERα and ERβ expression

In Experiment I, the phasic ERT decreased significantly CYP19 expression by 51% and ERα expression by 47%, whereas the small change in ERβ expression was not significant (II, Fig. 2A). In Experiment II, the tonic ERT increased significantly CYP19 expression by 69%, ERα expression by 86% and ERβ expression by 51% (II, Fig. 2B). In Experiment I, the phasic ERT decreased the ERα/ERβ ratio by 42% compared with the OVX group, but the ratio remained almost the same in OVX mice with or without tonic ERT (Experiment II). The correlations between measures of maze learning and hippocampal CYP19, ERα and ERβ gene expressions were also examined (II, Tables 3

and 4). The only significant correlation was found in Experiment II between RAM reference memory errors and ERα expression (II, Table 4), such that few reference memory errors in the RAM correlated with high levels of hippocampal ERα gene expression.

5.3. Effects of long-term ovariectomy and estrogen treatment on maze learning in aged mice

In the measures for RAM performance in Experiment I, the three-way ANOVA with 7- and 24-month-old mice combined revealed significant estrogen, operation, and age by operation interaction effects on the numbers of reference memory errors. Moreover, the age by treatment interaction also approached significance. When the working memory errors were analyzed, significant estrogen, age by operation and age by treatment interaction effects were found. Since age was a significant factor in all interactions, we continued the analysis separately for each age group. The analysis for the 7-month-old mice was previously published in (I) and is only summarized for comparison in Fig. 7. Among the 24-month-old mice, estrogen treatment decreased the number of reference memory errors similarly in SHAM and OVX groups (Fig. 7A) but had no effect on working memory errors in the RAM (Fig. 7B). An operation main effect was found on working memory errors, such that the sham-operated mice (SHAM and SHAM+E groups) performed worse than the OVX mice (OVX and OVX+E groups) (Fig. 7B).

In the T-maze, the three-way ANOVA revealed significant estrogen and age by treatment interaction effects and an almost significant effect of operation were found. We continued the analysis separately for each age group. The analysis for the 7-month-old mice was previously published in (I) and is only summarized for comparison in Fig. 7. In the 24-month-old mice, estrogen treatment and operation had no effect on number of correct choices in the T-maze (Fig. 7C). However, the treatment by operation interaction approached significance due to the improved performance of OVX+E mice and the slight impairment of SHAM+E mice. Whereas estrogen treatment had a major impact on maze learning in 7-month-old mice, its effects in 24-month-old animals were only marginal.

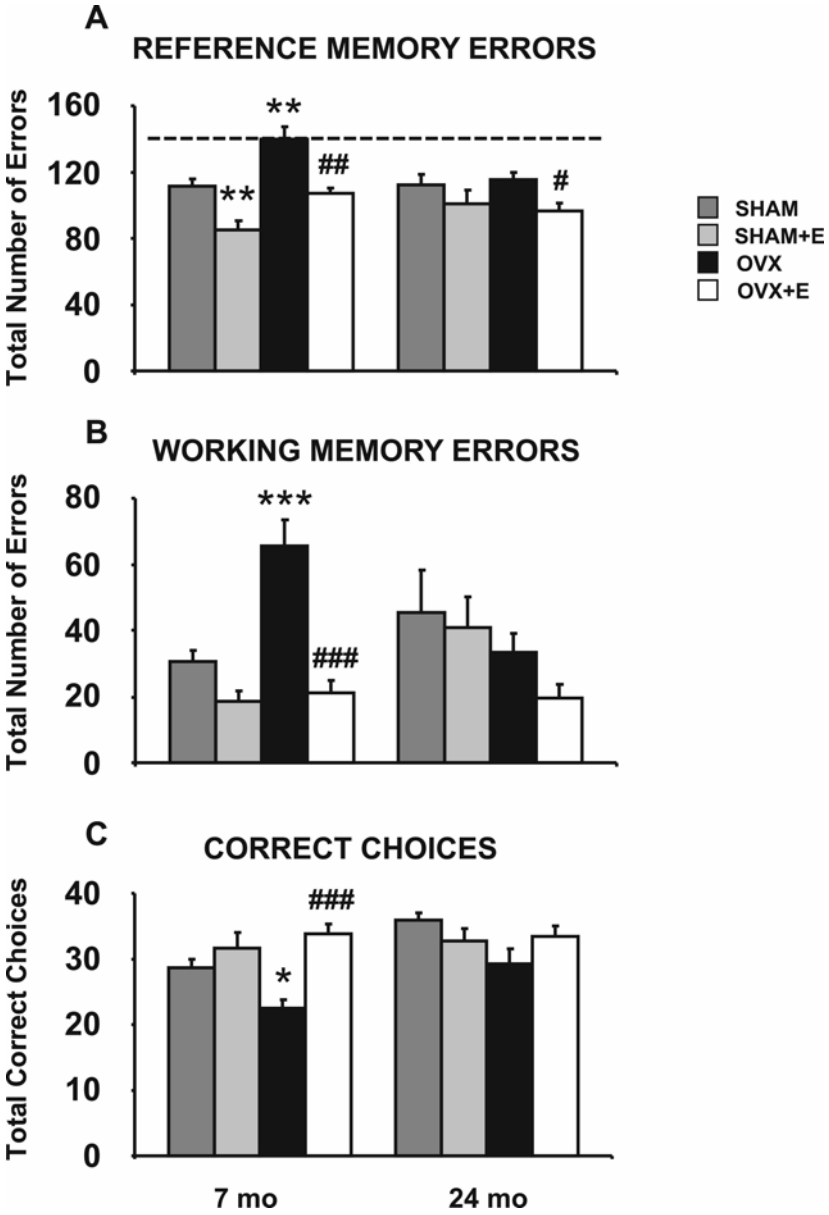

Figure 7. Effects of estrogen treatment on RAM and T-maze performance in 7-month-old and 24-month-old mice. The female OVX mice were treated with s.c. estrogen pellets (0.18 mg of 17β-estradiol) for 40 days (OVX + E and SHAM + E) before testing. Results are group means ± SEMs. The Y-axis scores for the RAM task are the total number of errors during 5 days, while those for the T-maze task are the total number of correct choices during 3 days. # significantly different from the OVX group, t-test (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$); * significantly different from the SHAM

group (* P < 0.05, ** P < 0.01, *** P < 0.001). The dashed line represents chance level in reference memory errors.

In the aging study (Experiment II), the numbers of reference and working memory errors were higher in OVX than in SHAM mice (III, Fig. 2). However, the ANOVA also revealed a highly significant operation by age interaction in working memory errors and marginally significant interaction in reference memory errors. Individual analysis of separate age groups showed that OVX increased the number of reference and working memory errors significantly only among 7-month-old mice but not among 11- and 24-month-old mice (III, Fig. 2A,B). While ovariectomy had a major effect on maze learning in 7-month-old mice, its effects in 11- and 24-month-old animals were only marginal.

5.4. Effects of estrogen treatment on spatial learning, hippocampal Aβ accumulation and plaque formation in AP mice

5.4.1. Findings in the memory tasks

The analysis of pooled T-maze data from all behaviorally tested age groups showed no overall difference in the number of correct choices between control and AP SHAM mice. Moreover, there was no genotype by age interaction (IV, Figs. 1A–C). However, a significant treatment effect and a treatment by age interaction were found. The subsequent post-hoc test revealed that AP OVX + E mice made significantly more correct choices than AP OVX mice. The treatment by age interaction likely derives from the finding that at the older ages (9 and 12 months; IV, Figs. 1B–C), the AP OVX mice performed worse than the other two groups, whereas at 6 months (IV, Fig. 1A), the three groups performed rather similarly.

In the combined data of the water maze, the ANOVA showed that AP SHAM mice had longer escape distances than control mice in both visible and hidden platform tasks (IV, Figs. 2A–C). In the visible platform task, no treatment effect was found on escape distance, whereas in the hidden platform task, the treatment effect and the treatment by age interaction approached significance. These effects are most likely derived from the robust impairment of the OVX group at 6 months of age (IV, Fig. 2A) and their slight impairment at 9 months of age (IV, Fig. 2B) since the groups behaved similarly at 12 months of age (IV, Fig. 2C). The spatial memory impairment in AP mice was confirmed

by the probe test revealing that AP SHAM mice spent less time in the target quadrant compared to control mice at the age of 12 but not at 6 or 9 months (Fig. 8A). However, there was no treatment effect on probe trial performance. At the age of 12 months, the spatial search bias of AP mice (all three treatment groups pooled) correlated negatively with both hippocampal total Aβ40 and Aβ42 levels (Fig. 8B). There was no difference between the genotypes in the swim speed (data not shown). In the visible platform subtest, no treatment effect was found on the swim speed (data not shown), whereas during hidden platform training, the treatment effect was significant (data not shown). The post-hoc test revealed that the AP OVX + E group was significantly slower than the AP SHAM group and nonsignificantly slower than AP OVX group.

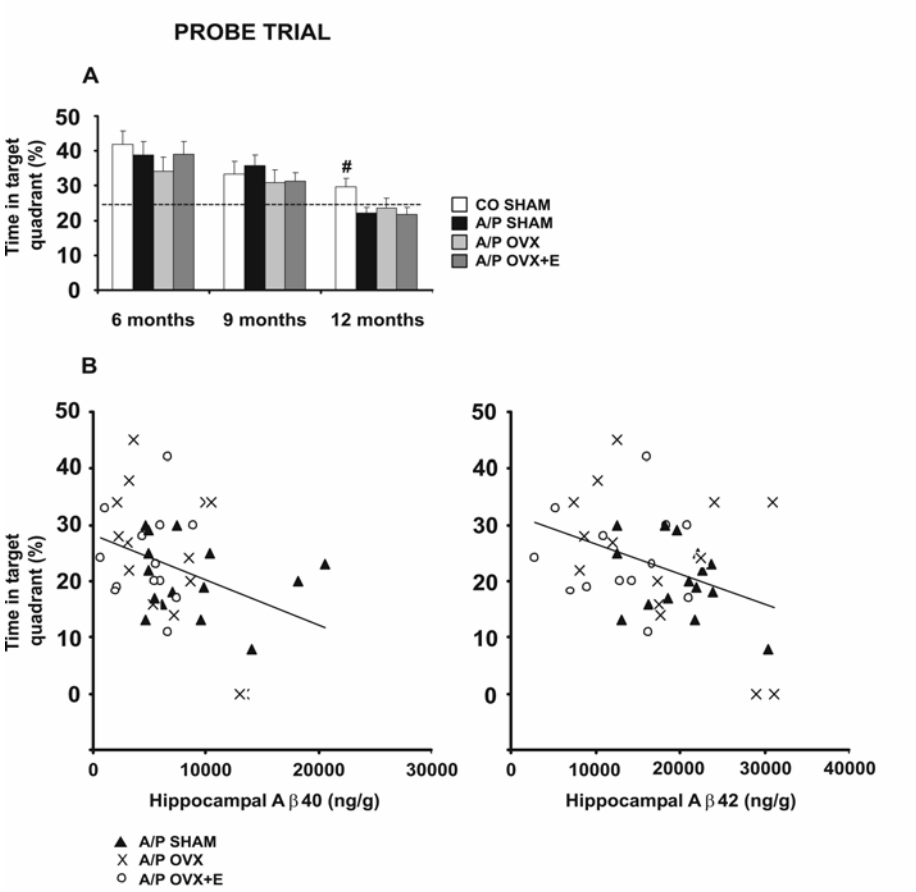

Figure 8. (A) Effects of genotype (control, CO vs. A/P), ovariectomy, and estrogen treatment on the probe trial performance of water maze. In time spent in target quadrant, the control mice performed better than A/P SHAM mice at the age of 12 months (# $p = 0.011$). Results are group means ± SEMs (the dashed line represents chance level). (B)

At the age of 12 months, the spatial search bias of A/P mice correlated negatively with both hippocampal total Aβ40 ($r = -0.36$, $p = 0.02$) and Aβ42 levels ($r = -0.39$, $p = 0.01$).

The number of working memory errors in the RAM was not affected by genotype (IV, Figs. 4A–C). However, control mice made more reference memory errors than AP SHAM mice (IV, Figs. 4D–F). There was no genotype by age interaction effect on the RAM performance. In the pooled analysis of all age groups, the treatment effect was significant for the reference memory errors and approached significance for working memory errors. These treatment effects are largely due to the superior performance of 6-month-old AP OVX + E mice compared to the two other groups (IV, Figs. 4A, D).

5.4.2. Hippocampal Aβ40 and Aβ42 levels and amyloid plaque counts

In AP mice, the amyloid levels increased dramatically with age. The 17-month-old AP SHAM mice had about 50 times higher hippocampal Aβ40 and 40 times higher Aβ42 levels than 6-month-old AP SHAM mice. The hippocampal Aβ40 and Aβ42 levels were below the detection level in all control mice at all ages. In the pooled data of all age groups, ovariectomy did not influence hippocampal levels of Aβ40 or Aβ42 when A/P SHAM and OVX mice were compared (IV, Table 1). No treatment by age interaction was found, indicating that there was a similar increase of Aβ40 and Aβ42 levels during aging in the SHAM and OVX groups. The comparison between AP OVX and estrogen-treated mice (both OVX + E and SHAM + E) revealed no effect of estrogen on the hippocampal levels of Aβ40 or Aβ42 (IV, Table 1). Moreover, the age-related increase in Aβ40 and Aβ42 levels was similar in OVX and estrogen-treated groups (no treatment by age interaction). Also, the separate t-tests between SHAM versus OVX and OVX versus OVX + E groups did not reveal differences in the Aβ40 and Aβ42 levels at any age. Finally, there were no differences in the Aβ40 and Aβ42 levels between SHAM and SHAM + E mice at 17 months of age (IV, Table 1).

The 17-month-old AP SHAM mice had about 15 times more hippocampal amyloid plaques than the 9-month-old AP SHAM mice. There were no differences in the plaque counts between the treatment groups (SHAM, OVX, and estrogen treatment) at the age of 9 or 17 months. Amyloid plaque counts between SHAM and SHAM + E mice at the age of 17 months did not differ (IV, Table 1).

6. DISCUSSION

6.1. Methodological considerations

6.1.1. The animal model

Traditionally rats have been used most often in behavioral assessment of learning and memory in rodents. During recent years use of mice has become more and more common, due to the increasing numbers of different kinds of gene manipulated mouse lines, offering tools to examine molecular mechanisms of leaning and memory, as well as the pathological hallmarks of neurological diseases. When the studies of this thesis were started, the goal was to accumulate basic knowledge of the possibilities of estrogen treatment to modulate the cognitive processes and characteristics in normal mice and the mice carrying AD-like pathology, namely the double transgenic APPswe and PS1(A246E) mice. These mice have elevated levels of the fibrillogenic Aβ42 in the brain and they develop amyloid plaques from the 9 months of age (Borchelt et al. 1997). The plaque formation starts from the subiculum, hippocampus and caudal cortex and then extends to other cortical areas, thus resembling the neuropathological changes occurring in early AD (Braak and Braak 1991). Therefore, the obvious choice was to first characterize the properties of estrogen treatment on cognition in normal mice, in order to appropriately evaluate the potency of estrogen on the same characteristics in transgenic mice.

6.1.2. Evaluation of ovariectomy and estrogen treatment

The objective and advantages of using OVX as a model for postmenopausal state in rodents have already been described above (see chapter 2.4.1.). Briefly, this operation leads to depletion of endogenous estrogen and, when performed on young or middle-aged rodents, ovariectomy represents a tool to study the physiological responses of estrogen depletion only, separated from those of the aging process that naturally are related to perimenopausal age. On the other hand, the postmenopausal state in women obviously includes the possible contributing of aging in cognition. Therefore, we wanted to examine the effects of OVX and ERT on learning and memory also in aged mice (III).

The continuous ERT produced by the s.c. estradiol pellets (I-IV) was chosen to resemble the chronic transdermal estrogen administration in postmenopausal women

ranging from weeks to months. For comparison, the phasic ERT (II) was chosen to reveal the effects of a treatment analogous to 'phasic' orally administered ERT. It is essential to acknowledge here that most often HRT in women consists of estrogen combined with progesterone. However, treatment with estrogen alone is not uncommon, and the aim of thesis was to determine the contribution of estrogen alone to cognitive performance.

6.1.3. Choice for hippocampal neurochemistry and ER and aromatase expression

As described above, estrogen has been shown to modulate the cholinergic and monoaminergic systems in the brain. Therefore, our aim was to elucidate the contribution of estrogen depletion and ERT to the measures of the metabolites of these neurotransmitter systems in the hippocampus and correlate them with the behavioral measures (I). To date, limited data is available about the contribution of the different ER subtypes, ERα and ERβ on learning and memory. In this study, the interest was on whether the gene expression measures of hippocampal ERs would correlate with the measures of learning in the RAM and T-maze. For gene expression measurements, we chose to take samples from the hippocampus, as this is the brain site (in addition to hypothalamus and cholinergic basal forebrain) with the largest reported number of estrogen receptors and which is most responsive to estrogen manipulation, and often the only target that has been evaluated as well. In addition to the ER measurements, we were interested to examine the effects of different kinds of estrogen manipulation also on hippocampal aromatase expression since brain is capable of synthesizing estrogen also locally. A key player in estrogen biosynthesis is the aromatase enzyme that is encoded by the cytochrome P450 19 (CYP19) gene, an enzyme converting androgens to estrogens. This enzyme is synthesized in neurons and astrocytes. Thus, we wanted to determine whether the effects of estrogen treatment could be mediated indirectly via local estrogen biosynthesis.

6.1.4. Choice of the memory tasks

The one-arm-baited version of RAM used in this study is not a commonly used version of the task. Most often four out of eight arms of RAM are baited to make the distinction between visits to never baited arms and re-entries to once baited arms. However, based on our previous study, the four-arms-baited RAM proved to be difficult for mice to learn, requiring several weeks of training (Ikonen and Riekkinen 1999). To allow

testing of same mice in more than one task during a one-month test period, we decided to modify the task so that it could be completed within a week. The working memory component of RAM is sensitive to hippocampal damage (Rissanen 1999), so this task was used as a hippocampal task, although including also the reference memory component which is presumably dependent on non-hippocampal, probably striatal functioning.

The position discrimination in the T-maze was chosen as a control task that, unlike RAM (I-IV) and water maze (IV), did not rely on the hippocampus for successful task completion. This task represents a typical reponse habit learning task, in which lesions to caudate-putamen have been shown to impair the performance (Oliveira et al. 1997).

The Morris water maze (IV) is a spatial memory task, consisting of two different components, the cued learning and the spatial learning. The cued version of the task is used as a control for the spatial version and evaluates the possible visual or motor impairments of the subject. The spatial task, in contrast, is based on the animal's ability to navigate to the platform using the spatial cues in the testing environment and it is a task which demands intact hippocampal functioning. Thus, it is a task reflecting the animal's ability to learn spatial relations and can be understood as a task detecting memory of a declarative nature.

6.2. Effects of ovariectomy and estrogen treatment on learning and memory in normal mice

The attempt to evaluate the effects of estrogen on such a complex phenomenon as learning and memory is a demanding task. First of all, estrogen alone exerts its effects throughout the body, and the effects on brain alone are very extensive. Therefore, the possible contribution of all the physiological effects – in addition to those related directly to learning and memory per se – possibly affecting the performance in the tests measuring learning and memory, must be carefully taken into consideration.

The main findings with the normal mice examined in this study were the contrasting effects of ovariectomy and estrogen treatment on maze learning. Ovariectomy impaired and estrogen treatment improved acquisition of the RAM task, not only in OVX mice but also in SHAM female and male mice. However, estrogen treatment improved

acquisition of the T-maze task only in OVX mice. This improvement induced by the estrogen treatment was even more pronounced in the mice with the longer, 40-day treatment than in the mice with the shorter, 7-day treatment. Also, five weeks of tonic ERT via the pellet implant decreased the amount of reference memory errors in RAM, whereas five weeks of daily injections of estrogen (20 microg) slightly impaired the performance in the T-maze.

Based on lesion studies, some, but not all, of estrogen effects found in the present study could be mediated by the hippocampus. Acquisition of the 1/8 RAM task employed in the present study as well as the more widely used 4/8 RAM task (Jarrard 1978) consists of two learning processes. The animal learns to avoid arms that remain nonbaited across days (as measured by so-called reference memory errors) and to avoid re-entries into visited arms (as measured by so-called working memory errors). In the 1/8 RAM task, mice learn quickly to avoid reentries into the same arms and seldom make working memory errors after the first 2 days of task acquisition. On the other hand, mice learn quite slowly to go straight to the only baited arm and visit on average three other arms before the baited one even on the fifth day of training. Like in the 8/8 and 4/8 -baited versions of the RAM, the number of working memory errors are increased by dorsal hippocampal lesion in mice in the 1/8 RAM (Rissanen 1999). On the other hand, hippocampal lesions in mice also have been shown to increase reference memory errors in the RAM version with 3/8 or 4/8 constantly baited arms but not with the 1/8 baited version employed in the present study (Cho and Jaffard 1995, Rissanen 1999). On the contrary, a previous study in our lab (Liu et al. 2002) showed that a fimbria-fornix lesion slightly improved acquisition of position discrimination in the T-maze. Therefore, the beneficial learning effects of chronic estrogen treatment are likely to be mediated also through other brain areas in addition to the hippocampus. One brain area possibly involved is the striatum, since damage to the dorsal striatum has been found to increase the number of reference memory errors in rats in the RAM (Colombo et al. 1989). Furthermore, position discrimination in T-maze, which can be considered as an egocentric memory task, requires intact functioning of the striatum (Oliveira et al. 1997).

There is some evidence that the preference for the strategy to complete a memory task may differ depending on the blood estrogen levels in female rodents (Korol and Kolo

2002). Many spatial tasks can be resolved by either a hippocampal dependent place strategy or a striatum dependent response strategy. The 1/8 RAM employed in this study presumably gives the animal an opportunity to complete the task successfully by using either one, whereas the T-maze task is more clearly a procedural, response learning task. Therefore, an attempt to couple the somewhat different findings in RAM and T-maze with each other could lead to a more comprehensive view of the effects of estrogen on cognition. Korol and her colleagues (2004), using a rotated T-maze task allowing the use of either a spatial or response strategy, found that rats with high estrogen levels outperform the estrogen deprived controls in tasks requiring an allocentric place strategy, whereas rats with low or very low estrogen levels prefer to use the procedural response strategy. Sava and Markus (2005) using a water maze task with either distant or local cues near the goal in the pool, examined the different learning capabilities of rats during different stages of the estrous cycle. They noted that the rats during estrus depend more on the cue near the goal whereas rats in proestrus use also the more distant cues when navigating to the goal. Thus, the dependence on the local cue could suggest the use of a response learning strategy whereas the use of a wider range of cues could be evidence for a spatial strategy. Together, these examples would suggest that the hippocampus may be more sensitive to estrogenic manipulation than the striatum.

According to our measurements, after two weeks of pellet implantation the serum estradiol levels were 136 pg/ml and five weeks after the implantation 65 pg/ml on average (II, Table 4). This implies that, in contrast to the information given by the manufacturer (IRA, Sarasota, FL, USA), these pellets do not deliver estradiol at a constant level, but the amount of delivered estradiol is higher at the beginning of the treatment and slowly declined towards the end of the treatment. However, the serum estradiol concentrations in the OVX mice treated with estradiol-pellets in this study appeared to be at the level of the proestrus stage in normal intact mice (Grasso and Reichert 1996). Thus, this can be interpreted as a high physiological concentration, since during the estrus stage, the serum levels have been reported to be approximately 25 pg/ml. Thus, when extrapolated to the study by Korol and Kolo (2002) it could be argued that the OVX mice having continuous ERT in our study should be using preferably the spatial, hippocampal strategy when performing the memory task. Indeed, this is the case in terms of the hippocampus-based working memory component in

RAM, since especially the longer, 40 day ERT improved the performance compared to control mice. The results of this study in terms of the T-maze and RAM reference memory component of RAM performance of the pellet treated mice, are not in agreement with the results by Korol and Kolo (2002). Namely, in this study, chronic ERT decreased the number of reference memory errors in RAM and also improved the T-maze performance, factors presumably dependent merely on enhanced procedural learning. However, one striking difference between the studies can be found in the form of estrogen administration. Korol and Kolo used two daily estradiol injections, administered 21 days after OVX, 48 and 24 hours prior to testing. This kind of acute treatment may cause very different effects on cognitive performance than the chronic treatment used in our study. Indeed, Galea et al. (2001) found that long-term treatment with daily 10 μg injections of estradiol, given to OVX rats four hours prior to testing, impaired not only hippocampus dependent learning, as indicated by the increased number of working memory errors in RAM, but also a striatum-based cued win-stay task. Their injection protocol produced blood estrogen levels of about 120 pg/ml, which is almost twice the level occurring in proestrus. Interestingly, our study is partly in agreement with the study of Galea et al., since the OVX mice receiving phasic ERT via daily injections of 20 mg were non-significantly impaired in the T-maze and the blood estrogen levels in these mice were also very high (~1200 pg/ml) around the time of testing.

Collectively, the difference between tonic and phasic ERT on maze learning in the present study is largely in agreement with previous studies. ERT administered via the pellet decreased significantly the number of reference memory errors in the RAM and also tended to improve maze learning in general compared with OVX controls. In contrast, estrogen injection proved to be ineffective and a 20-microg injection of estrogen even impaired the T-maze performance of OVX mice. Rissanen et al. (1999), employing a similar pellet ERT as used in this study, also found that this kind of ERT improved the performance of OVX mice in Morris water maze, another spatial memory task. In contrast, Fugger et al. (1998) using ERT at 20 microg injections reported no improvement in the same water maze task. Furthermore, Miller et al. (1999) found that OVX mice treated with an estrogen pellet performed better in a spontaneous alternation in T-maze, compared with OVX mice. Collectively, these studies suggest that not only

the estrogen dose, but also the pattern of administration can have a major impact on the effects of estrogen on learning and memory.

6.3. Estrogen, hippocampal neurotransmitters and memory

In this study, we showed that in mice ovariectomy per se leads to decreased hippocampal ChAT activity when compared to sham-operated mice. However, it was somewhat surprising that estrogen treatment had no effect on hippocampal ChAT activity in either OVX or sham-operated mice. This result is partly in contrast with earlier data showing that estrogen treatment increases the number of ChAT-immunoreactive neurons in certain areas of the basal forebrain in both OVX mice (Miller et al. 1999) and OVX rats (Gibbs 1997). The OVX time before estrogen treatment in our study was unusually long, 4 months (in the 7-day treatment groups), so it is possible that the cholinergic system had become less responsive to estrogen after such a long duration of OVX. On the other hand, our results with the 40-day treatment are concordant with a study showing that continuous, long-term (4 weeks or 6 months) estrogen treatment given to OVX female rats had no effect on hippocampal ChAT activity or high affinity choline uptake (Gibbs 2000a). Furthermore, the fact that no effect of estrogen treatment on ChAT activity was detected in males is consistent with previous reports (Luine and McEwen 1983, Luine 1985). In the present study, ovariectomy led to decreased levels of hippocampal NA and DOPAC but not DA. As far as NA is concerned, this finding is in agreement with an earlier study in which ovariectomy was reported to reduce the hippocampal NA and DA levels in C57BL/6 mice (Toriizuka et al. 1999). In rats, however, 27-day estrogen treatment given to OVX animals had no effects on the hippocampal NA and DA levels (Luine et al. 1998). In contrast to some earlier reports (Archer et al. 1988, Pisa et al. 1988) we found no significant correlation between the hippocampal NA, DA, or DOPAC and behavioural performance in the RAM or T-maze. On the other hand, DA or DOPAC levels in the basal ganglia have been reported to correlate with position discrimination in the T-maze (Taghzouti et al. 1985, Tanila et al. 1994).

With respect to the hippocampal neurotransmitters, the serotonergic system was most clearly affected by the estrogen treatment. The concentrations of hippocampal 5-HT and 5-HIAA appeared to depend on the duration of the estrogen treatment and ovarian status. In sham-operated mice, the 7-day estrogen treatment led to an increase in the 5-

HIAA/5-HT ratio, whereas the ratio after the 40-day treatment returned to a level between the values of these two groups. However, in OVX mice, the 7-day estrogen treatment caused a notable, though nonsignificant, decrease in the hippocampal 5-HIAA/5-HT ratio. Furthermore, in the OVX mice, the 40-day estrogen treatment led to a decrease in the hippocampal 5-HT while a less significant decrease was observed in the sham-operated mice after the 7 days treatment. Ovariectomy has been reported to affect the hippocampal 5-HIAA/5-HT ratio in rats so that after 2 weeks but not 4 weeks after OVX there is a decrease in the ratio (Zhang et al. 1999). The different effect of estrogen on serotonin turnover in sham-operated vs OVX mice may be mediated by the presence vs absence of progesterone. Namely, in rats, levels of progesterone have been found to correlate negatively with hippocampal 5-HT concentrations during pregnancy (Glaser et al. 1990). However, Luine et al. (1998), who measured brain monoamines in nontreated OVX rats and OVX rats treated with estradiol for 28 days, found no treatment effects on the hippocampal 5-HT levels. The differences in the levels of hippocampal monoamines between the Luine et al. study in rats and our study in mice could be explained by the different durations of the estrogen treatment and OVX. Although depletion of brain serotonin does not affect performance in the RAM working memory task, performance of the same task correlates with hippocampal 5-HT levels (Luine et al. 1990). Further, although hippocampal damage does impair acquisition of the position discrimination task in the T-maze, task performance correlates with the hippocampal 5-HIAA levels in aged female rats (Tanila et al. 1994). However, we did not find any significant correlation between maze learning performance and the serotonergic parameters.

6.4. Estrogen, hippocampal ERα and ERβ expression and memory

Correlations between measures of gene expression and spatial learning revealed interesting details (II, Tables 3 and 4). In a measure of spatial learning, shown to be sensitive to estrogenic manipulation in this study (the reference memory component of the RAM) the correlations between cognitive performance and ERα expression were significant; i.e. the fewer reference memory errors the mice made, the higher the ERα expression in the hippocampus. Notably, this effect was significant only in OVX mice and OVX mice treated with tonic ERT. On the other hand, no significant correlations were found between behavioral measures and ERβ expression. These observations seem

to disagree with the findings in ERα and ERβ knockout mice. Indeed, Fugger et al. (1998) claimed that ERα activation would impair spatial navigation in the water maze in female mice, whereas Rissman et al. (2002) reported that ERβ was required for female mice to optimally complete the same task. However, when comparing those results with the present findings, it should be borne in mind that the water maze task used in the abovementioned studies is believed to rely on unimpaired hippocampal function, whereas the reference memory component of RAM is also strongly dependent on striatal memory functions (Oliveira et al. 1997). In this light, it is interesting to note that the dominant ER subtype in the female C57BL/6J hippocampus is ERα (Mitra et al. 2003). Still, the studies conducted with ERα or ERβ knockout mice should be interpreted cautiously, since the lack of one of the ER subtypes throughout the brain may affect indirectly performance in the cognitive task by changing noncognitive behaviors.

Given the possibilities for choosing either a hippocampal or a striatal strategy to complete RAM, it is possible that the OVX mice receiving continuous, stable estrogen treatment switched to using the place (i.e. hippocampal) strategy, which could account for the decreased number of reference memory errors compared with non-treated OVX mice. Therefore, the correlation between RAM learning and ERα mRNA expression in the hippocampus could reflect a direct interaction between these two parameters. Nevertheless, this does not necessarily mean that the critical site of action is exclusively the hippocampus, as ERα expression may simultaneously be elevated in several other brain structures. One good additional candidate target for estrogen actions is the cholinergic projections from the basal forebrain to the hippocampus and cortex. These projections are necessary for estrogen-mediated effects on hippocampal connectivity (Lam and Leranth 2003), and there is evidence that estrogen can influence the cholinergic projections and this effects can vary as a function of estrogen dose and regimen (Gibbs and Aggarwal 1998). Furthermore, a considerable proportion of the cholinergic neurons projecting to the hippocampus express ERα (Miettinen et al. 2002).

The findings of this study indicate that ERT not only affects brain by directly delivering estrogen across the blood–brain barrier, but also indirectly regulates the estrogen synthesis in the brain by modulating the aromatase gene expression. Furthermore,

estrogen has been reported to stimulate aromatase enzyme activity without affecting mRNA levels, pointing to posttranscriptional effects of estrogen on aromatase activity levels (Roselli et al. 1997). Whether the direct estrogen delivery to the brain or the modulation of aromatase activity is more important for the outcome of ERT remains to be explored in further studies.

6.5. Estrogen, aging and memory

Long-term (40 days) estrogen treatment in aged, 24-month-old female mice had varying effects on maze learning depending on the memory task. It decreased the number of reference memory errors in the win-stay RAM task, but had no effect on the number of working memory errors, and marginally improved performance of OVX mice in the position discrimination task in the T-maze. These estrogen effects were in the same direction (towards improved performance) but much less robust than observed in young female mice in this study, suggesting that old age ora a long time period without the ovarian hormones makes the brain less responsive to estrogen. A comparison of these results with our findings in 7-month-old female mice that were subjected to exactly the same treatment protocol and behavioral tests (I, II) reveals interesting age-related changes in the effects of ERT and OVX. In the young adult female mice, long-term (40 days) estrogen treatment reduced both reference and working memory errors in sham-operated and OVX mice (I). In contrast, in aged female mice, the estrogen-induced improvement was significant only in the reference memory component of the task. In addition, the effect of OVX on maze learning was age-dependent. Thus, it seems that both long-term ovariectomy and long-term estrogen treatment lose some of their beneficial effects as the female mice reach the post-estropausal age.

A study using 5- and 22–24-month-old mice found that in both age groups, estrogen treated OVX mice were improved in an object recognition test, a non-spatial recognition memory task (Vaucher et al. 2002). In that study the OVX was conducted and the of s.c. estradiol capsules were implanted 21 days prior to behavioral testing. Also in contrast to the lack of estrogen effect on working memory in our 24-month-old mice, Miller et al. (1999) reported that 25-month-old OVX mice treated with 17β-estradiol for 30 days showed improved performance compared to non-treated OVX mice in spontaneous alternation in the T-maze, a working memory task. However, the mice used by Miller et al. (1999) and Vaucher et al. (2002) had been ovariectomized for only 3 or 4 weeks,

whereas in our study the mice had been ovariectomized for as long as 19 months before behavioral testing. In addition, we used a slightly longer duration of estrogen treatment (40 days). Furthermore, the behavioral tasks used in this study require food deprivation whereas the spontaneous alternation task used by Miller et al. (1999) and the object recognition test (Vaucher et al. 2002) do not, which together with differences in OVX and ERT durations, might account for the discrepant findings. Frick et al. (2002) found that daily s.c. injections of 5 mg of β-estradiol-3-benzoate, started 5 days before Morris water maze testing, improved the performance of aged (27–28-month-old) intact female mice in the spatial version of the task. These findings are in agreement with this study in terms of ERT-induced improvement of SHAM-mice in the reference memory component of RAM since the Morris water maze can also be considered as a spatial reference memory task. Thus, the spatial reference memory of intact post-estropausal mice can be improved by estrogen delivered in a variety of treatments, regimens and durations.

Gibbs (2000b) investigated the cognitive effects of long-term ovariectomy and different durations of continuous estrogen (E2) or weekly estrogen plus progesterone (E2 + P) treatments in a delayed matching-to-position (DMP) task in the T-maze in aged rats. The rats were OVX at the age of 13 months and tested at the age of 22–25 months. The E2 or E2 + P treatment started immediately or 3 months after OVX significantly improved the performance of OVX rats. However, the hormone therapies had no effect on DMP performance if the treatments were started after a long OVX duration (10 months). Previously, Gibbs (1999) has also shown that young OVX rats having 2 months of continuous estrogen treatment were improved in the DMP task. The aged rats with the hormone treatment starting 10 months after ovariectomy and 6–8 weeks before the DMP testing are comparable to the estrogen treated mice in this study. In summary, these results, together with our findings, suggest that estrogen treatment might have a beneficial effect on working memory also in aged OVX rodents but only if the estrogen treatment has been started shortly after OVX.

Partly dissimilar effects of OVX and ERT on working memory errors in the RAM task between young adult and aged mice may indicate that by 24 months of age, general age-related degenerative changes had rendered the hippocampus unresponsive to estrogen. Recent findings support this assumption also at the synaptic level. For example,

estrogen treatment increases the density of hippocampal dendritic spines in young OVX rats but this effect is no longer observed in aged OVX rats (Adams et al. 2001). It has been hypothesized that in aged rats, the decreased responsiveness of the hippocampus to estrogen treatment is attributable to the reduced number of synaptic ERα (Adams et al. 2002). Conversely, the relatively similar OVX and ERT effects on both T-maze performance and reference memory errors in RAM in both young adult and aged mice may indicate that the striatum remains responsive to estrogen until a much higher age than the hippocampus. Indeed, chronic estrogen treatment has been shown to reverse OVX-induced decrease in locomotor activity and striatal dopamine release in adult Sprague–Dawley rats (Ohtani et al. 2001). Furthermore, in aged OVX mice MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced striatal dopamine depletion could be reduced by estrogen treatment (Miller et al. 1998). Therefore, it is possible that in aged mice, estrogen treatment might enhance dopamine-mediated striatal functions leading to improved performance in some cognitive tasks, presumably in certain aspects of spatial reference memory.

One interesting finding in this study was the somewhat surprising uterine response to estrogen treatment in sham operated and OVX mice. In contrast to findings with 7-month-old female mice in this study (I), the estrogen-induced uterine growth in aged mice was more pronounced in sham-operated than in OVX mice. This increased responsiveness is probably due to the fact that in aged mice the number of uterine estrogen receptors is increased compared to young mice (Xu and Clark 1990). Whereas in 7-month-old female mice the improved RAM performance correlated with increased uterine weights (I), no such correlation was found in the aged mice of this study. This correlation suggests that in young adult mice, the responsiveness of the brain to estrogen may be related to overall physiological responsiveness to estrogen. Conversely, it seems that brain and peripheral responses to estrogen become decoupled at the post-estropausal age in female mice. Given the findings in this study (II) showing that tonic, long-term ERT increases the hippocampal aromatase expression in young adult mice, it is tempting to postulate that the different balance between brain and peripheral estrogen effects in aged mice compared to young mice (II) could be explained by a decline in the aromatase activity in the aged female brain. Examining this question would be interesting in future studies by evaluating the aromatase expression and behavioral outcome using animals of different ages.

Yet another interesting finding between the study with 24-month-old mice and our previous one with 7-month-old mice (I) was that the pooled group of aged OVX mice (OVX and OVX + E groups) performed better in the working memory component of the RAM compared to the pooled group of sham-operated mice (SHAM and SHAM + E groups), whereas among the 7-month-old mice the difference between OVX and sham-operated groups was in the opposite direction, i.e. the sham-operated mice outperformed the OVX mice. This poses the question of whether OVX conducted at an early age contributes differently on learning and memory in young mice compared to mice that are closer to their estropausal age.

To address this question we tested another pre-estropausal group of OVX and sham-operated mice without ERT (11 months of age with OVX duration of 8 months) in the RAM and compared these mice with the OVX and sham-operated mice of 7 and 24 months of age. From this experiment we found that the effect of OVX on the number of reference and working memory errors was highly age-dependent. Notably, this difference was mainly due to impaired performance of the OVX group of 7-month-old mice, whereas there were no differences between OVX and sham-operated groups in either 11-month-old or 24-month-old mice. The difference in the OVX effect between 7- and 11-month-old mice (with OVX duration of 4 vs. 8 months, respectively) indicates that the impairment in spatial memory induced by an early-age OVX can be observed in young adult mice but diminishes when the mice approach their estropausal age. It might also indicate that the long duration of ovariectomy rather than aging per se was one of the factors that made the OVX mice resemble more the sham-operated mice of advanced age, even though the mice were tested at different ages. This observation is consistent with the findings of Frick et al. (2000) showing that spatial reference memory impairment occurs earlier in females than in males, suggesting a role for declining estrogen levels (or lack of cycling estrogen levels) in this phenomenon. It is also in agreement with the observation of Gibbs (2000b) that the brain response to estrogen treatment declines between 3 and 10 months after ovariectomy. Therefore, ERT begun early after estropause may alleviate or prevent impairment in spatial learning in aging females, but may well be less effective when started at a late post-estropausal age.

6.6. Estrogen, Aβ accumulation and memory in AP mice

The present study investigated the effects of long-term estrogen deprivation (3–14 months) and estrogen treatment (3 months) on brain Aβ levels, amyloid plaque counts, and cognitive functions in transgenic AP mice. Neither an overall analysis across all ages nor separate analyses at each age point showed any significant effect of ovariectomy or estrogen treatment on hippocampal Aβ levels or amyloid plaques. In behavioral testing, estrogen treatment in ovariectomized AP mice dramatically improved position discrimination in the T-maze and decreased the number of reference memory errors in the RAM. However, estrogen treatment had only a marginal effect on the performance of the AP OVX mice in the cued and spatial versions of water maze (WM). These results show that the estrogen treatment in a transgenic mouse model of AD improves performance in the same learning and memory tasks as in the normal C57BL/6J mice. However, the estrogen effects in these mice appeared to be unrelated to Aβ-induced cognitive deficits. These results do not support the concept that estrogen treatment decreases the risk or alleviates the symptoms of AD by inhibiting the accumulation of Aβ or formation of amyloid plaques.

The present results showing unaltered brain Aβ levels after long-term ovariectomy (3–14 months) are clearly in conflict with some previous studies. There are several factors that may explain the discrepancy between the studies. Differences in the genetic background of the mice are an unlikely explanation since the mice used in the studies of Levin-Allerhand et al. (2002) and Levin-Allerhand and Smith (2002) had the very same APPswe transgene construct in the same strain (C57BL/6) as used in our study. In contrast, the age at ovariectomy and the duration of ovariectomy appear to have a major impact on the results. Namely, the most impressive effects of long-term ovariectomy (3–4 months) on Aβ levels (50–130% increase) were seen in mice ovariectomized at the age of 4–5 weeks (Levin-Allerhand and Smith 2002, Zheng et al. 2002), whereas a similar duration of ovariectomy increased Aβ levels only by 20–30% in mice ovariectomized after sexual maturity (Zheng et al. 2002). However, it seems that estrogen depletion for 6–8 weeks has no effect on amyloid levels even if the ovariectomy is done at a very young age (4 weeks) (Levin-Allerhand et al. 2002). Based on previous studies, at least 10 weeks of estrogen depletion are needed to detect any increase in Aβ levels (Levin-Allerhand and Smith 2002, Petanceska et al. 2000, Zheng

et al. 2002). However, in this study, Aβ levels were not increased even in mice which had been ovariectomized for as long as 14 months. Therefore, based on the present and previous results, it can be argued that long-term estrogen depletion (>3 months) may increase Aβ production during the early stages of amyloid pathology, but has no effect on the aggregation, rapid accumulation, and deposition of Aβ into plaques at later stages. From this perspective, the results presented here do not support the hypothesis that the increased risk of AD in women 20 years after menopause can be attributed to faster production of Aβ as a result of to the estrogen depletion. However, a definite answer to this question may only be found using postmortem neuropathological analyses in female AD patients that have participated in controlled trials with or without ERT.

In accordance with our previous studies in male AP mice (Liu et al. 2003, Puoliväli et al. 2002), female AP mice showed a task-specific memory impairment. They were impaired in WM, but interestingly, outperformed control mice in the reference memory component of RAM and performed similarly in the T-maze. One explanation for the task differences might be that our T-maze and RAM tasks rely on different memory functions than the WM task. Thus, fimbria-fornix transection (rendering the hippocampus dysfunctional) in mice has no effect on the position discrimination learning in the T-maze or on the total number of errors in the current win-stay version of the RAM task (Liu et al. 2002), which leads us to assume that performance in these tasks, excluding working memory component of RAM, relies mainly on brain functions distinct from the hippocampus, presumably the striatum (Oliveira et al. 1997). On the other hand, mice with fimbria-fornix lesions are impaired in both the hidden and visible platform versions of the WM (Liu et al. 2002), evidence in favor of the dependence of this task on hippocampal function. Thus, the observed pattern of task-specific impairment is fully consistent with the known pathology in the AP mice: a marked and early accumulation of amyloid plaques in the dorsal hippocampus but few plaques in the striatum (Liu et al. 2002). In addition, consistent with earlier findings in male mice (Liu et al. 2003, Puoliväli et al. 2002), we also found that the WM retention deficit in female AP mice correlated with the levels of hippocampal Aβ42, favoring a direct link between hippocampal Aβ accumulation and WM spatial memory impairment in our mice.

Long-term estrogen treatment in ovariectomized AP mice dramatically improved position discrimination in the T-maze. This improvement was most prominent in the 9- and 12-month-old mice, i.e. after longer durations of OVX. Furthermore, it was more attributable to impaired performance of OVX mice than an improvement of OVX + E mice compared to SHAM mice. In addition, estrogen treatment decreased the number of reference memory errors in the RAM. These results are in agreement with our findings in 7-month-old C57BL/6J mice, where chronic (40 days) estrogen treatment improved the performance of OVX mice in both position discrimination in the T-maze and the same win-stay version of the RAM as used in the present study (I). However, unlike in ovariectomized C57BL/6J mice (I), estrogen treatment did not significantly decrease working memory errors in AP OVX mice in the present study. Chronic estrogen treatment had only marginal effect on the performance of the AP OVX mice in the cued and spatial versions of WM. This finding appears to be in conflict with a previous study on WM acquisition reporting improved learning in the hidden and in the cued versions of the task after 2 weeks of estrogen treatment in ovariectomized C57BL/6J mice (Rissanen et al. 1999). However, the present water maze data are in agreement with the Rissanen et al. (1999) study in that ovariectomized 6-month-old AP mice were dramatically impaired in spatial navigation compared with sham-operated AP mice. Interestingly, whereas only long-term ovariectomy appears to impair position discrimination in the T-maze, impairment in spatial navigation is present only shortly after the ovariectomy and declines with time. These contrasting time-dependent effects of OVX are also consistent with the concept that these two tasks tax different memory systems.

The present study revealed an almost complete diametric dissociation between the effect of AP genotype and estrogen treatment in different learning tasks. The most consistent learning impairment in sham-operated AP mice compared to sham-operated control mice appeared in the hidden platform version of the WM, in which no estrogen effect was found. Conversely, the estrogen treatment effect was most dramatic in the position discrimination task in the T-maze, in which the AP genotype had no effect, and in the reference memory component of win-stay RAM task, in which the AP mice even outperformed the control mice. This leads to the conclusion that the effects of Aβ accumulation and estrogen treatment on learning and memory do not interact. The separation of these effects can occur either at the molecular level, so that Aβ and

estrogen affect different intracellular signaling pathways, or alternatively at the systems level, so that their influence is modulated via separate neural circuits. The current findings suggest that the effects of estrogen are most pronounced in tasks relying mainly on striatal functions (Oliveira et al. 1997), whereas amyloid pathology in AP mice primarily affects hippocampal functions.

The present study was designed to represent the situation in clinical trials with postmenopausal women at a risk of AD or with patients already suffering from AD. We observed that long-term estrogen treatment did not inhibit or decrease hippocampal Aβ accumulation, and did not restore the hippocampal dysfunction in WM, apparently induced by increased Aβ accumulation in the hippocampus of AP female mice. However, the amyloid pathology in AP mice did not prevent estrogen from having similar beneficial effects on certain learning and memory functions mediated through other brain structures, as it has in normal C57BL/6J mice (I). Our results suggest that ERT in patients with already diagnosed AD is likely to be ineffective, and thus are in agreement with recent placebo-controlled clinical trials failing to show any cognitive improvement in female AD patients after prolonged ERT (Henderson et al. 2000, Mulnard et al. 2000, Thal et al. 2003, Wang et al. 2000). Extrapolated to humans, this study further emphasizes the risk of assessing possible effects of estrogen on the pathophysiology of AD based on its effects on cognition alone. On the other hand, whereas estrogen replacement may not offer a way to slow down the amyloid-induced neuronal dysfunction, it may still have beneficial effects on cognitive performance of AD patients, at least by preventing some, albeit mild, cognitive impairment due to the sudden decline in the estrogen levels after the menopause.

7. CONCLUSIONS

The results of this study demonstrate that in adult female mice chronic estrogen treatment improves acquisition of different maze tasks that tax different brain areas and systems and this also occurs to a certain degree in male mice. Estrogen treatment influenced also the hippocampal serotonin turnover (I).

This study also demonstrated that the pattern of estrogen delivery may dramatically change the ERT effect on the brain. Tonic ERT had beneficial memory effects and increased the level of CYP19 and ER alpha gene expression, whereas phasic ERT though having the same peripheral effect had the opposite influence on the brain. This finding indicates that ERT not only affects the brain by directly delivering estrogen across the blood–brain barrier, but also indirectly regulates the estrogen synthesis in the brain by modulating the aromatase gene expression. This finding suggests that effects on the brain of peroral estrogen may also differ from the effect of transdermal estrogen in humans (II).

The effects of ERT on cognitive performance in aged mice were in the same direction (towards improved performance) but much less robust than those seen in young female mice, suggesting that old age or a long time duration without exposure to ovarian hormones makes the brain less responsive to estrogen. Therefore, ERT begun early after estropause may alleviate or prevent impairment in spatial learning in aging females, but be less effective if started at a late post-estropausal age (III).

In female AP mice, ERT improved the performance in the same learning and memory tasks as in the normal mice. However, the estrogen effects in these mice appeared to be unrelated to the Aβ-induced cognitive deficits. These results do not support the claim that estrogen treatment decreases the risk or alleviates the symptoms of AD by inhibiting the accumulation of Aβ or the formation of amyloid plaques (IV).

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APPENDIX: ORIGINAL PUBLICATIONS (I–IV)

I

Effects of ovariectomy and estrogen treatment on learning and hippocampal neurotransmitters in mice

Heikkinen T., Puoliväli J., Liu L., Rissanen A., Tanila H.

Hormones and Behavior 2002, 41: 22-32.

II

Effects of estradiol on spatial learning, hippocampal cytochrome P450 19, and estrogen alpha and beta mRNA levels in ovariectomized female mice

Iivonen S.*, Heikkinen T.*, Puoliväli J., Helisalmi S., Hiltunen M., Soininen H., Tanila H.

Neuroscience 2006, 137: 1143–1152.

* both authors contributed equally to this work

III

Effects of long-term ovariectomy and estrogen treatment on maze learning in aged mice

Heikkinen T., Puoliväli J., Tanila H.

Experimental Gerontology 2004, 39: 1277-1283.

IV

Estrogen treatment improves spatial learning in APP+PS1 mice but does not affect beta amyloid accumulation and plaque formation

Heikkinen T., Kalesnykas G., Rissanen A., Tapiola T., Iivonen S., Wang J., Chaudhuri J., Tanila H., Miettinen R., Puoliväli J.

Experimental Neurology 2004, 187: 105-117.

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SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

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