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Cholinergic Neurons of the Rodent Basal Forebrain and Their Content of Estrogen Receptor Alpha

Doctoral dissertation

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ABSTRACT

The cholinergic system of the basal forebrain plays a pivotal role in cognitive functions such as arousal, attention, learning and memory. In aging, a decline in sensory and motor performance can be accompanied by cognitive deficits. Gradual deterioration of cognitive abilities and development of memory impairment are essential criteria to characterize dementia. Most demented individuals are victims of Alzheimer's disease (AD). The deterioration of the cholinergic function has been recognized as one of the key factors in the AD etiology.

The prevalence of AD was reported to be higher among women than among men. Loss of gonadal hormones in women after the menopause is believed to contribute to the development of AD. Therefore, hormone or estrogen replacement therapies (ERT) were considered to play a significant role in AD prevention. This hope was based on *in vitro* and *in vivo* studies that showed a wide range of estrogenic effects on the survival, structure and function of neurons. In addition, estrogen was shown to influence the formation of beta-amyloid (Aβ) that is an indispensable feature of AD pathology. The findings that the cholinergic neurons of the basal forebrain express estrogen receptor alpha (ERα) provided an anatomical substrate for the estrogen action on this neurotransmitter system.

In the present study we aimed to investigate whether the modulation of estrogen status affects the number of cholinergic neurons in the basal forebrain nuclei, their content of ERα and Aβ accumulation in rodents. The numbers of choline acetyltransferase (ChAT)-immunoreactive (ir) neurons of adult rats, aged mice and transgenic AD mouse model were estimated using stereology. The results revealed that the depletion of estrogen upregulates the percentage of ChAT-ir cells that contain ERα-ir in aged mice. Moreover, the number of ChAT-positive neurons containing ERα-ir in the cell nucleus was significantly lower at 12 months than at 6 months of age. Neither ovariectomy nor ERT affected Aβ plaque counts in transgenic mice of AD.

The results of this series of studies suggest that changes in estrogen status influence the presence of ERα in the cholinergic neurons of the rodent basal forebrain even at old age. Furthermore, age *per se* could be a detrimental factor that independently from estrogen status modulation and genetic background regulates the intracellular distribution of ERα in mice. This knowledge is important for future therapeutic strategies targeting ERs and its intracellular transport factors.

National Library of Medicine Classification: WT 155, WP 522
Medical Subject Headings: Alzheimer disease/etiology; prosencephalon; neurons; neurotransmitter agents; estrogens; estrogen receptor alpha; amyloid beta-protein; choline o-acetyltransferase; rats; mice; animals, genetically modified

To Valentina and Augustina

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Kuopio, November 2005

Giedrius Kalesnykas

ABBREVIATIONS

ANOVA	one-way analysis of variance
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
APP/PS1	APPswe + PS1 (A246E)
APPswe	mutated amyloid precursor protein, Swedish mutation
Aβ	beta-amyloid
cAMP	cyclic adenosine monophosphate
ChAT	choline acetyltransferase
CV	coefficient of variance
DAB	3,3'-diaminobenzidine
ER	estrogen receptor
ERT	estrogen replacement therapy
GABA	gamma-aminobutyric acid
HACU	sodium-dependent high affinity choline uptake
HDB	horizontal diagonal band of Broca
HRT	hormone replacement therapy
Hsp90	heat shock protein 90
mRNA	messenger RNA
MS	medial septum
MSVDB	medial septum-vertical diagonal band of Broca
M1-5	muscarinic acetylcholine receptor, subtypes 1-5
NbM	nucleus basalis magnocellularis (in primates: nucleus basalis of Meynert)
NGF	nerve growth factor

NGS	normal goat serum
OVX	ovariectomy
OVX+E	ovariectomy with 17β-estradiol treatment
PS1	presenilin-1
PS2	presenilin-2
PB	phosphate buffer
$p75^{NTR}$	low-affinity neurotrophin receptor p75
SHAM	sham-operated
SERM	selective estrogen receptor modulator
TBS	Tris buffered saline
trkA	high-affinity neurotrophin receptor
VDB	vertical diagonal band of Broca
WHI	Women's Health Initiative (study)

LIST OF ORIGINAL PUBLICATIONS

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

I Miettinen RA, Kalesnykas G, Koivisto EH. Estimation of the total number of cholinergic neurons containing estrogen receptor-alpha in the rat basal forebrain. Journal of Histochemistry & Cytochemistry 50(7): 891-902, 2002.

II Kalesnykas G, Puoliväli J, Sirviö J, Miettinen R. Cholinergic neurons in the basal forebrain of aged female mice. Brain Research 1022(1-2): 148-156, 2004.

III Heikkinen T, Kalesnykas G, Rissanen A, Tapiola T, Iivonen S, Wang J, Chaudhuri J, Tanila H, Miettinen R, and Puoliväli J. Estrogen treatment improves spatial learning in APP + PS1 mice but does not affect beta amyloid accumulation and plaque formation. Experimental Neurology 187(1): 105-117, 2004.

IV Kalesnykas G, Roschier U, Puoliväli J, Wang J, Miettinen R. The effect of aging on the subcellular distribution of estrogen receptor-alpha in the cholinergic neurons of transgenic and wild-type mice. European Journal of Neuroscience 21(5): 1437-1442, 2005.

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1. Introduction

The cholinergic system of the basal forebrain consists of four overlapping cell groups: medial septum (MS), vertical (VDB) and horizontal (HDB) limbs of the diagonal band of Broca and nucleus basalis magnocellularis (NbM). The common feature of all cholinergic neurons is their content of acetylcholine as neurotransmitter and its synthesizing enzyme choline acetyltransferase (ChAT). The brain cholinergic system is involved in a number of cognitive functions including arousal, attention as well as learning and memory. In Alzheimer's disease (AD), the selective loss of cholinergic neurons and their cortical markers (Arendt et al., 1983; Davies and Maloney, 1976; Whitehouse et al., 1981; Whitehouse et al., 1982; Bowen et al., 1976; Perry et al., 1977) are the most consistent and severe neurochemical deficits. Furthermore, a correlation was shown between the reduction of cortical cholinergic markers and cholinergic cell loss in the NbM and *pre mortem* mental status scores in individuals having senile dementia (Perry et al., 1978). After these findings, AD was hypothesized to be a disorder of one neurotransmitter system similarly as Parkinson's disease. This led to the development of drugs that could alleviate cholinergic deficits. However, later studies showed that AD is far more complex than it was thought before. Although, the cholinesterase inhibitors were able to enhance cholinergic neurotransmission, the treatment had rather modest effect on the disease progression. New treatment strategies for AD became necessary. For many years estrogens were known as "female" hormones that produce their main effects in the female reproductive tissues. However, later on, the use of tritium-labeled steroid hormones revealed estrogen-containing cells in the brain (Pfaff and Keiner, 1973). Moreover, a wide distribution of estrogen receptor alpha (ERα) and ER beta (ERβ) was found throughout the rostral-caudal extent of the brain and spinal cord (Shughrue et al., 1997). In parallel, emerging evidence from experimental *in vivo* and *in vitro* studies suggested multiple effects of estrogens on cells. Estrogens were shown to have neurotrophic (Toran-Allerand et al., 1999), neuroprotective (Dubal et al., 1999; Hawk et al., 1998), anti-apoptotic effects (Brusadelli et al., 2000; Garcia-Segura et al., 1998; Garnier et al., 1997), antioxidative properties (Behl et al., 1997; Green et al., 1998) and even have the ability to inhibit the formation of toxic beta-amyloid (Aβ) (Jaffe et al., 1994; Xu et al., 1998). Furthermore, the higher incidence rate of AD among women than men was demonstrated in population-based prospective cohort studies (Andersen et al., 1999; Fratiglioni et al., 2000). The decline in estrogens' levels in postmenopause was considered one of the main risk factors contributing to the memory decline in women. In light of multiple positive findings from experimental studies, estrogen (ERT) and hormone (HRT) replacement therapies were thought to have a potential to delay the progression

of memory disorders at old age. However, the findings from epidemiological data were inconsistent with such conclusions (Hogervorst et al., 2000). Finally, the first large-scale double blinded clinical trial reported findings where the benefit of taking ERT was outweighed by the increased risk of stroke, myocardial infarction and venous thrombolism in healthy postmenopausal women (Rossouw et al., 2002).

At the time this study was started, there was an overall optimism regarding the pharmacological properties of estrogen actions in the central nervous system. Therefore, this study was designed to evaluate the effects of estrogen status on the number of cholinergic neurons in the rodent basal forebrain using immunohistochemistry. The goal of this study was also to investigate the presence of ERα in cholinergic neurons. In order to get an unbiased estimation of neuronal numbers, the stereological method was applied. Different animal models were used in this study: adult rats and mice, aged mice, and a transgenic mouse model of AD.

2. Review of the literature

2.1 The cholinergic system of the central nervous system

2.1.1 Anatomy of the cholinergic system

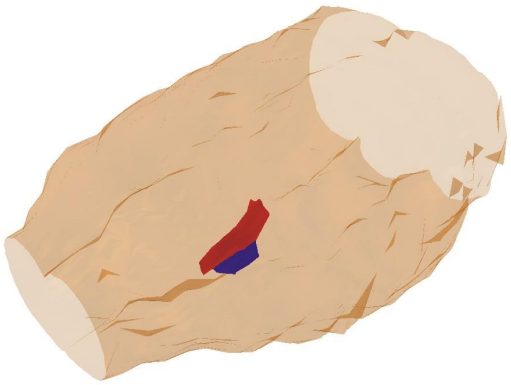
The presence of cholinergic neurons in the basal forebrain was originally reported by Shute and Lewis in 1967 and later confirmed by others (Härtig et al., 2002; Semba, 2000; Zaborszky et al., 1999). In the early 1990's, Mesulam introduced a *Ch* classification to designate the groups of cholinergic neurons (Mesulam et al., 1983a,b). As this classification was based on the topographical variations of cholinergic cell groups and their particular cortical and subcortical targets, it has been widely used. The cholinergic system is divided into eight groups of cholinergic cells (*Ch1-Ch8*). At the most rostral level of the basal forebrain, cholinergic cells are located in the MS and VDB nuclei. These cell groups are designated as *Ch1* and *Ch2*. In rodents, 30-50% of MS and 50-75% of VDB cells are cholinergic (Mesulam, 1994; Wainer and Mesulam, 1990), whereas in primates and humans these percentages are 10 and 70, respectively (Mesulam et al., 1983b; Mesulam, 1994; Mufson et al., 1989). The strip of cells that extends towards a horizontal axis and is situated ventrolaterally to the *Ch2* constitutes the nucleus of HDB or *Ch3*. In rodents, 10-20% of cells are ChAT-positive (Wainer and Mesulam, 1990). In primates, only 1-2% of cells can be described as cholinergic (Mesulam, 1994). The neocortex and amygdala as well as reticular nucleus of thalamus are innervated by the *Ch4* group of cells that is found within the NbM (Mesulam, 1994). That is the largest group of cholinergic neurons of the basal forebrain in rodents, primates and humans. Approximately 90% of cells in NbM are cholinergic (Mesulam, 1994; Wainer and Mesulam, 1990). Immunohistochemical and *in situ* hybridization studies showed that the main constituent of the non-cholinergic part of basal forebrain cholinergic nuclei is a gamma-aminobutyric acid (GABA)-containing population of cells (Gritti et al., 1994; Smith and Booze, 1995; Semba, 2000).

The *Ch5-Ch8* groups of cholinergic cells are situated in the brainstem. The *Ch5* and *Ch6* groups of cells are located in the pedunculopontine tegmental and laterodorsal tegmental nuclei, respectively. In human brain nearly all (approximately 90%) neurons situated in the pedunculopontine nucleus (*pars compacta*) are ChAT-immunopositive. Remaining neurons are mainly catecholaminergic in that they are tyrosine hydroxylase-immunopositive. The laterodorsal tegmental nucleus has relatively pure proportion of ChAT-positive cells with small amounts of

GABAergic, glutamatergic and catecholaminergic neurons (Mesulam, 1994; Wainer and Mesulam, 1990; Tohyama and Takatsuji, 1998). The medial habenular nucleus contains the *Ch7* group of the cholinergic neurons. Approximately 80-90% of cells in the parabigeminal nucleus also stain for ChAT and are defined as *Ch8* group (Mesulam, 1994; Wainer and Mesulam, 1990).

In terms of functional neuroanatomy, all cholinergic nerve cells described above are projecting neurons. In addition, some ChAT-containing interneurons were reported in the caudate-putamen nucleus, nucleus accumbens, olfactory tubercule and islands of Calleja complex, cerebral cortex, olfactory bulb and hippocampus (Butcher, 1995; Lauterborn et al., 1993; Oh et al., 1992; Zaborszky, 2002). The precise function of these cells is largely unknown.

A



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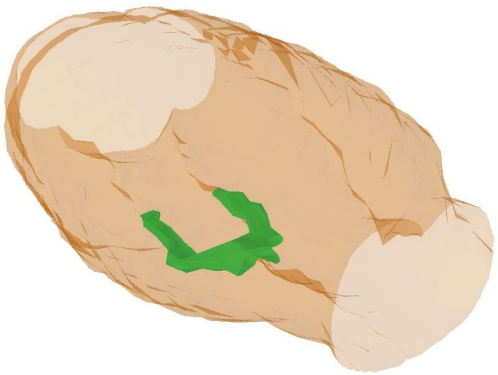

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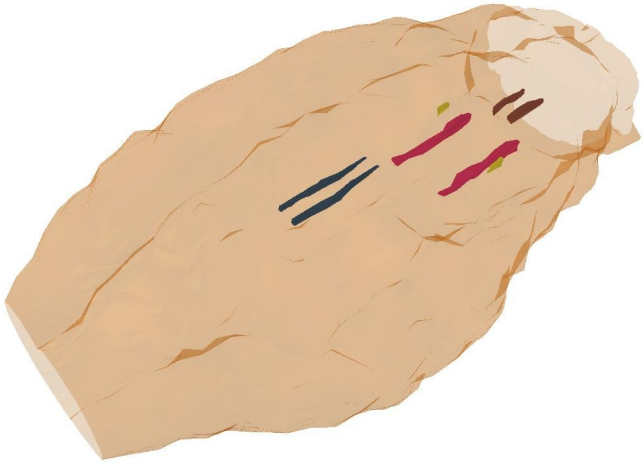


Figure 1. Schematic drawing of the cholinergic nuclei in the rat basal forebrain: (A) medial septum (red color) and the vertical diagonal band of Broca (blue), (B) horizontal diagonal band of Broca, (C) nucleus basalis magnocellularis, (D) pedunculopontine tegmental (red), laterodorsal tegmental (yellow), medial habenular (black), and parabigeminal (brown) nuclei. The drawings were made using Neurolucida software (MicroBrightField Inc., USA) for serial section reconstruction with the aid of a rat brain atlas (Paxinos and Watson, 1998).

2.1.2 Target areas of cholinergic innervation

Brain areas that are innervated by cholinergic neurons were revealed using tract-tracing methods in rodents and primates combined with acetylcholinesterase (AChE) enzyme histochemistry and ChAT immunohistochemistry. Due to ethical restrictions, these types of experiments could be not applied to study cholinergic innervation in the human brain. However, data on *post mortem* human tissue indicated that the organization of the cholinergic innervation in humans and non-human primates is largely identical (Mesulam, 1994).

The most rostral parts of the cholinergic cell groups in the basal forebrain, *Ch1* and *Ch2*, innervate the hippocampus (Mesulam, 1994; Wainer and Mesulam, 1990). AChE-rich cholinergic fibres are seen within CA2, CA3 and CA4 regions of the hippocampal proper, in the inner part of the molecular layer of the dentate gyrus, and in the subiculum. *Ch3* provides the major source of cholinergic innervation to the olfactory bulb. Cerebral cortex receives cholinergic innervation from the largest group of cholinergic cells in the basal forebrain that is situated in the NbM and is referred to as *Ch4*. The human *Ch4* complex is subdivided into 6 sectors: anteromedial, anterolateral, anterointermediate, intermediodorsal, intermedioventral, and posterior. Different *Ch4* parts project to different cortical areas. Studies in the monkey brain revealed that anteromedial part provides the major source of cholinergic innervation to medial cortical areas including the cingulate gyrus; anterolateral-to the frontoparietal region and the amygdaloid nuclei; intermediodorsal together with intermedioventral-to the laterodorsal frontoparietal, peristriatal and midtemporal regions; and posterior-to the superior temporal and temporopolar areas (Mesulam, 1994). Despite major differences in the overall density of cholinergic axons among different cytoarchitectonic areas, the cholinergic innervation of primary sensory and unimodal association areas is weaker than that of the paralimbic and limbic areas. Cholinergic cortical innervations also display some target layer specificity. Taken together, the density of cholinergic axons is higher in layers I, II as well as the upper parts of layer III in the cerebral cortex (Mesulam, 1994).

Both *Ch5* and *Ch6* send their main projections to the thalamus (Mesulam, 1994). In addition, there is evidence that *Ch5* and *Ch6* may innervate also the cerebral cortex, basal forebrain, and extrapyramidal structures such as the striatum, globus pallidus, subthalamic nucleus and substantia nigra. In summary, the functional distinction between these nuclei could be formulated as follows: *Ch5* more participates in sensory processing and extrapyramidal motor control, whereas *Ch6* is more closely related to the limbic system (Mesulam, 1994).

2.1.3 Acetylcholine and the cholinergic synapse

In contrast to most known neurotransmitters acetylcholine (ACh) is not a derivative of the amino acid metabolism (Bear et al., 2001). ACh is derived from acetyl coenzyme A which is a ubiquitous product of cellular respiration in mitochondria, and choline, which plays an important role in fat metabolism and is transported to the brain both free and in phospholipid form via blood (Bear et al., 2001). ACh synthesis requires a specific enzyme, ChAT, which is synthesized on ribosomes located in the soma of neurons and transported to the axon terminal. The transmitter ACh, in turn, is synthesized by ChAT in the cytosol of the axon terminal, and concentrated in synaptic vesicles by the vesicular acetylcholine transporter (VAChT; Erickson et al., 1994; Weihe et al., 1996). After ACh is released into synaptic cleft as a result of an action potential, it binds to ACh receptors that can be located on both pre- and post-synaptic membranes. Remaining ACh is removed from the synaptic cleft by a specialized enzyme, AChE. AChE converts ACh into acetic acid and choline, which is returned back to the presynaptic cell by a reuptake process.

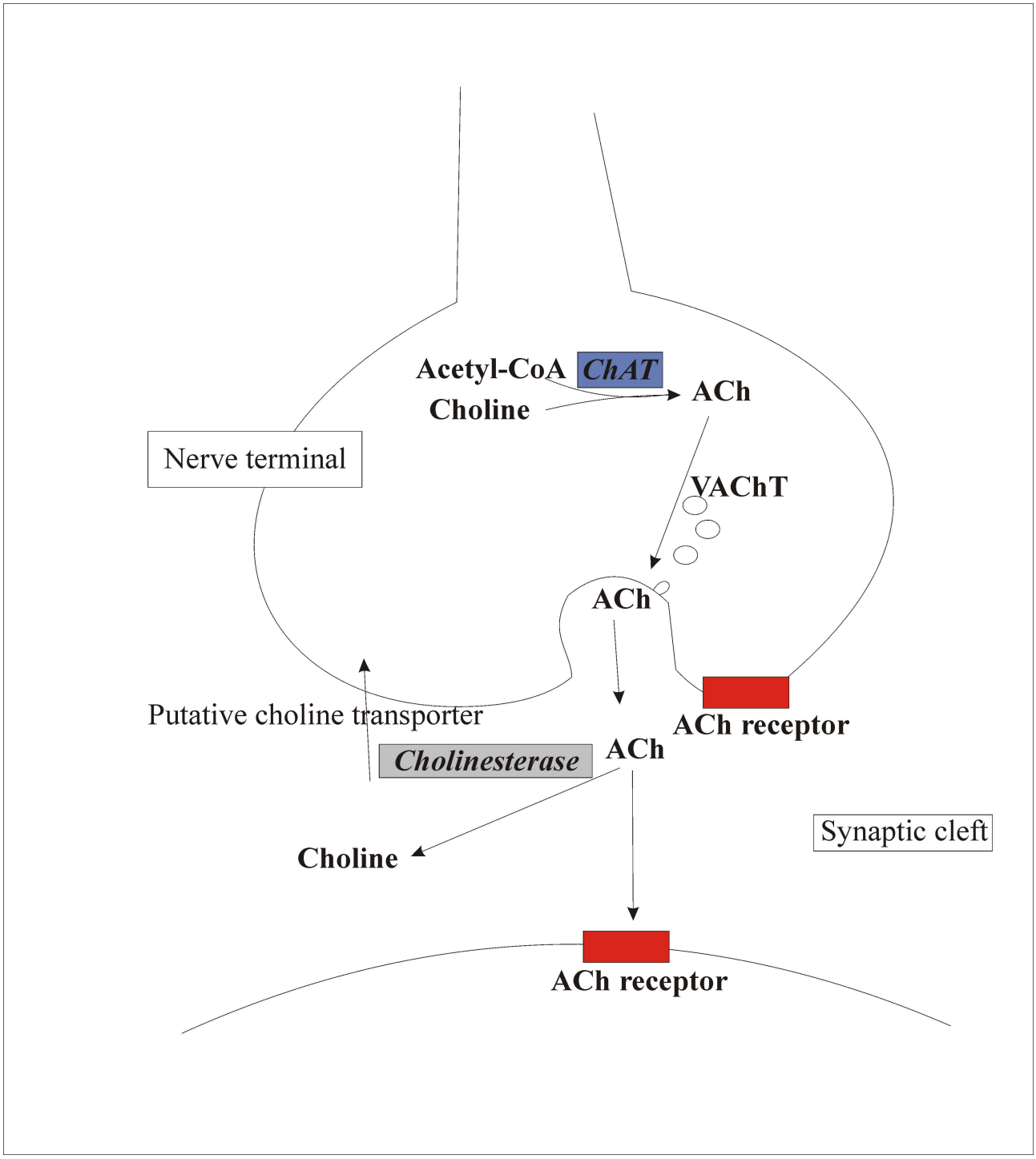


Figure 2. Schematic drawing of the cholinergic nerve terminal. Ach, acetylcholine; ChAT, choline acetyltransferase; VAChT, vesicular acetylcholine transporter (adapted from Oda, 1999).

2.1.4 Acetylcholine receptors

Acetylcholine receptors in the mammalian nervous system are divided into two groups: muscarinic and nicotinic. They were named based on the ability of the natural alkaloids, nicotine and muscarine, to mimic the effects of ACh as a neurotransmitter.

Muscarinic receptors are coupled to G proteins and either act directly on ion channels or are linked to a variety of second-messenger systems (Ehlert et al., 1994). They predominate in the mammalian cerebral cortex. To date, five muscarinic receptors (M1-M5) have been identified using molecular biology techniques. It is unclear whether a specific subtype of muscarinic receptors represents a unique function. However, it is known that stimulation of muscarinic receptors M1, M3 and M5 activates different ion channels as well as phospholipases (A2, C and D). That eventually leads to activation of different second messenger systems. The activation of muscarinic M2 and M4 receptor subtypes reduces the levels of cyclic adenosine monophosphate (cAMP) through the inhibition of adenylate cyclase (Ehlert et al., 1994; Felder, 1995). Muscarinic receptors can be located on both pre- and post-synaptic membranes. Furthermore, approximately 30% of pyramidal neurons of the cerebral cortex of layers III and V display immunoreactivity for both muscarinic and nicotinic ACh receptor subtypes (Schröder et al., 1989). Although different muscarinic receptor subtypes are present throughout the whole brain, their proportions vary in different regions. For example, in the cerebral cortex, M1 receptors are more numerous than M2. Additionally, M1 receptors density is at its highest in the limbic area and the association cortices. In contrast, M2 is more prevalent in primary sensory and motor areas of cortex (Mash et al., 1988). Furthermore, M1 receptors constitute 40-60% of all muscarinic receptor subtypes in the neocortex and the hippocampus; M2 is predominant in the basal forebrain, midbrain, medulla, pons region and cerebellum, whereas M4 is most abundant in the corpus striatum (Ehlert et al., 1994; Felder, 1995). M3 and M5 receptors are expressed at low levels in the brain.

Nicotinic cholinergic receptors belong to the group of ligand-gated ion channel receptors. They are composed of four different subunits (α, β, δ and γ), with a stoichiometry of two α subunits and one each of the other three subunits. In addition, multiple isotypes of each subunit type exist which are the products of individual genes. Therefore, there are a large number of possible subunit subtype compositions for nicotinic ACh receptor. This is reflected in the fact that at least nine different functional nicotinic receptors have been identified. They are expressed in cerebral cortex, thalamus, hippocampus, hypothalamus, interpeduncular nucleus and the superior colliculus (Arneric et al., 1994). However, little is known regarding the physiological role of most of these receptors in the central nervous system.

2.1.5 Functional implications of the cholinergic system

The cholinergic neurotransmission covers broad aspects of cortical function because of intense cholinergic innervation from the *Ch1-Ch4* groups. Acetylcholine may exert complex effects in the cerebral cortex. For example, it may have an inhibitory role directly or through the mediation of GABA-containing interneurons, or affect the cholinoceptive cortical neurons by causing a prolonged reduction of potassium conductance, which, in turn, makes cholinoceptive neurons more susceptible to excitatory inputs (Mesulam, 1994). In terms of behavior, cholinergic neurotransmission is involved in arousal, learning and memory, mood, reward and aggressive behavior. Experimental studies demonstrated that lesions of *Ch1-Ch4* cell groups or systemic administration of cholinergic antagonists may disrupt learning and memory processes (Mesulam, 1994). In addition, according to Buzsaki (1989), cholinergic innervation plays a major role in switching from on-line attentive processing, characterized by hippocampal theta rhythm, to an off-line period of consolidation, which is characterized by sharp wave activity.

2.1.6 Neurotrophin receptor expression in the cholinergic neurons

The nerve growth factor (NGF) was first described by Levi-Montalcini and Angeletti (1963) as an important trophic factor in the development and maintenance of noradrenergic peripheral sympathetic neurons. Later studies showed that NGF may increase ChAT levels in the cholinergic perikarya *in vitro* (Hefti, 1986; Martinez-Serrano et al., 1995) and increase ChAT activity in the basal forebrain, hippocampus, neocortex and neostriatum *in vivo* (Gnahn et al., 1983). Under normal conditions, the highest levels of NGF are present in the target fields of the basal forebrain cholinergic neurons: cerebral cortex, hippocampus and olfactory bulb (Conner and Varon, 1992; Conner et al., 1992). Immunohistochemical studies on primate and human material revealed that NGF is present in the cholinergic neurons of the basal forebrain (Mufson et al., 1994; Mufson et al., 1995) and is retrogradely transported from cholinergic cortical terminal to the perikarya where it may exert its function via NGF receptors. Two classes of NGF cell surface receptors have been found: (1) the low-affinity neurotrophin receptor with a molecular weight of 75 kDa ($p75^{NTR}$), and (2) the high-affinity transmembrane glycoprotein having a cytoplasmic protein kinase domain (trkA; Bothwell, 1991). Colocalization experiments in non-human primates and humans showed that 68-73% of all basal forebrain cholinergic neurons coexpressed both $p75^{NTR}$ and trkA receptors. Furthermore, trkA was found in 23-28% of ChAT-immunopositive neurons, whereas 4% of all basal forebrain cholinergic neurons co-expressed $p75^{NTR}$, but not trkA (Kordower et al., 1994). The

functional role of $p75^{NTR}$ receptor is to recruit NGF to the trkA receptor. The trkA receptor has a capability to activate cellular responses to NGF alone (Riccio et al., 1997).

A body of evidence suggests that NGF and estrogen systems interact within the basal forebrain cholinergic neuron populations (Muir, 1997). For example, significantly greater levels of ChAT (Loy and Sheldon, 1987) and $p75^{NTR}$ (Kornack et al., 1991) were detected during early postnatal development in female than in male rats. Furthermore, estrogen receptors are colocalized with the low-affinity $p75^{NTR}$ in the cholinergic cells (Toran-Allerand et al., 1992). This suggests the potential trophic effects of estrogens that could be reflected in the developmental differences of NGF receptor expression in the cholinergic neurons of the basal forebrain.

2.2 Cholinergic system in aging and Alzheimer's disease

2.2.1 Cholinergic system in aging

Animal studies. The available data on age-related changes in the cholinergic markers or neuronal counts from the basal forebrain of rodents are inconclusive. Discrepancies between data in several studies may result from the various methodological and animal species differences. This may also be compounded by other factors such as different age, gender and strain. For example, a reduction in size and number of ChAT/NGF receptor-positive cells in the basal forebrain during aging was reported in one study (Fischer et al., 1992), while swelling of ChAT-immunopositive neurons and no significant changes in the cholinergic cell numbers of MS and NbM during aging were observed by other group (Armstrong et al., 1993). Although both these studies were conducted on rats, the animals were from different inbred strains. Similarly, inconsistent findings were reported from other cholinergic parameters as well. Significant age-related reductions in ChAT activity of frontal and cerebral cortices were observed in aged rodents (Sarter and Bruno, 1998). However, changes in ChAT activity during aging might also be sex-dependent. Luine et al. (1986) showed that ChAT and AChE activity may differentially decrease in aged male and female rats than that in young ones.

More consistent results have been reported from sodium-dependent high affinity choline uptake (HACU) studies. HACU shows the ability of cortical cholinergic synapses to absorb choline. As a matter of fact, HACU is the rate-limiting step in ACh synthesis. Therefore, this marker reflects the functional activity of the cholinergic system. Experimental studies showed that HACU could remain unaltered during aging in rodents (Lebrun et al., 1990; Meyer et al., 1984; Sirviö et al., 1988).

Human studies. The analysis of cholinergic markers in human brains *post mortem* has also led to contradictory findings. A significantly decreased number of cholinergic cells in the basal forebrain (Whitehouse et al., 1982) and of cholinergic neurons in the NbM (De Lacalle et al., 1991) or a decrease in the cortical ChAT activity (Davies and Maloney, 1976) were demonstrated by some studies, while other groups reported an unchanged number of cholinergic cells in the NbM (Bartus et al., 1982; Chui et al., 1984) during aging. However, it is necessary to consider that the earliest age-related changes may occur at the cellular level and be expressed as a loss of cell volume or number of terminals. Such changes were reported to occur in aged animals. Mesulam et al. (1987) showed neuronal shrinkage in aged mice despite the unaltered number of basal forebrain cholinergic neurons. However, the size of neurons or number of their terminals was not investigated in aged human material. Furthermore, it is not known whether preclinical state of AD, known as mild cognitive impairment is temporally linked to a further decrement in cholinergic transmission that could be influenced by the AD pathology alone, but not by age *per se*. These questions should be addressed in future studies on aging and the cholinergic system.

2.2.2 Alzheimer's disease

Nearly one hundred years ago, the German neuropathologist and psychiatrist Alois Alzheimer first described cerebral atrophy, presence of extracellular neuritic plaques and intracellular neurofibrillary tangles as neuropathological hallmarks in the brain of a demented patient. Further studies revealed that these neuropathological changes occur initially in the medial temporal lobe structures such as the entorhinal cortex and hippocampal formation. At later stages, the pathology extends into other cortical and subcortical regions such as the basal forebrain cholinergic system (Bondareff et al., 1994; Braak and Braak, 1991; Geula, 1998).

The etiology of AD is heterogeneous. About 50% of early-onset familial AD individuals, which accounts for 4-8% of all AD cases, have mutations in three genes: presenilin-2 (PS2) on chromosome 1, presenilin-1 (PS1) on chromosome 14, and amyloid precursor protein (APP) on chromosome 21 (Selkoe, 1991). Additionally, apolipoprotein E is a well established risk factor for AD, which is found on chromosome 19 (Meyer et al., 1998). Recently, interleukin-1α has also been identified as a risk factor, which is associated with an earlier onset of AD (Grimaldi et al., 2000). Other genetic risk factors that could contribute to the early- as well as late-onset AD development are under investigation, e.g., nicastrin and ERβ (Helisalmi et al., 2004; Pirskanen et al., 2005).

2.2.3 Cholinergic system in Alzheimer's disease

In 1974, Drachman and Leavitt demonstrated that the blockade of the cholinergic receptors in young healthy individuals produces a memory deficit, which is similar to that seen in AD patients (Drachman and Leavitt, 1974). Subsequently, a severe loss (up to 95%) of cholinergic markers in the cerebral cortex in AD subjects was independently reported by two research groups (Bowen et al., 1976; Davies and Maloney, 1976). Later studies showed significant decreases (of varying extents, ranging between 15% and 95%) in the number of cholinergic neurons in the NbM of AD patients (Arendt et al., 1983; Geula and Mesulam, 1996; Iraizoz et al., 1991; Whitehouse et al., 1982). Furthermore, the severity of the cholinergic deficits in AD was found to positively correlate with the severity and duration of the AD (Francis et al., 1999; Perry et al., 1981). This encouraged the development and introduction of pharmacotherapies that would involve the cholinergic system modulating agents such as inhibitors of AChE (Orgogozo, 2003). However, the enthusiasm that cholinergic therapy may be used to eliminate memory and cognitive deficits in demented patients soon decreased. Clinical trials using these cholinergic drugs showed only modest improvements and could not restore cognitive function (for review see Trinh et al., 2003). There are several factors that could influence such an outcome. First, cholinergic degeneration is not apparent in cases with mild cognitive impairment (Davis et al., 1999). These individuals are the main target group for the disease prevention. Moreover, there is no general brain cholinergic system lesion in AD (Mesulam, 2004). The cholinergic nuclei in the brainstem remain relatively intact in contrast to the basal forebrain cholinergic neurons co-expressing $p75^{NTR}$. Finally, catecholaminergic neurons show even more prominent losses in activity at early stages of the disease (Zarow et al., 2003) than cholinergic cells. Therefore, the current treatment strategies that use cholinomimetics at preclinical or early stages of the disease might prove to be productive when combined with other therapeutic approaches than when used alone.

2.2.4 Experimental animal models used to study Alzheimer's disease and the cholinergic system

<u>Lesions of the cholinergic nuclei of the basal forebrain in experimental animals.</u>

The hypothesis that cholinergic dysfunction may lead to the development of cognitive disturbances facilitated a development of animal models that could mimic the loss of cholinergic function which is observed in AD patients. The selective injury of cholinergic nuclei using excitotoxins was believed to have the same effects on cognition of animals as in AD subjects. Indeed, the behavioral deficits are present in animals following cholinergic immunolesion (for review see Muir, 1997).

However, it was not known whether memory deficits were caused by lesion of cholinergic neurons or other types of cells (e.g. GABA) that are also located in cholinergic nuclei. Immunolesioning with the immunotoxin 192 IgG-saporin that selectively kills $p75^{NTR}$-bearing cholinergic projection neurons in the rat basal forebrain (Wiley, 1992) revealed that selective lesion of the septal area produces no memory deficits (Baxter et al., 1996; Berger-Sweeney et al., 1994; Torres et al., 1994). Furthermore, lesion models of animals are too restricted in terms of lesion place and are too acute to mimic AD, where cholinergic deterioration occurs gradually (Mufson and Kordower, 2001). Thus, the usefulness of such animal models to study pharmacological agents that combat AD pathology is questionable (Muir et al., 1993).

Transgenic animal models of AD. After the identification of AD-causing gene mutations, steps were taken to develop transgenic animal models of AD. These animals, in form of gene knockouts or insertion of wild-type and mutant transgenes, were supposed to mimic AD pathophysiology more accurately than animal lesion models. Indeed, AD transgenic mouse lines (see Table 1) show some features of human AD pathology. For example, the deposition of Aβ plaques (Borchelt et al., 1997; Holcomb et al., 1998), a modest loss of neurons (Calhoun et al., 1998; Takeuchi et al., 2000), loss of synaptophysin staining (Games et al., 1995) or deficits in long-term potentiation maintenance (Chapman et al., 1999) have been reported in transgenic mice containing various genes that have mutations associated with human AD. Furthermore, single APP and double APP/PS1 transgenic lines show behavioral impairments (Chapman et al., 2001). Robust changes in ChAT and AChE activity in both the neocortex and the hippocampus were described in double APPswe/PS1dE9 mice (Savonenko et al., 2005). However, the correlations between cholinergic markers and episodic-like memory parameters did not reach a corrected significance level. A recently developed triple transgenic mouse model of AD shows a progressive development of Aβ-containing plaques and hyperphosphorylation of the microtubule-associated protein tau resulting in tangles deposits in the neocortex and hippocampus (LaFerla and Oddo, 2005; Oddo et al., 2003).

Taken together, the transgenic mice modeling aspects of AD were demonstrated to be suitable for studies on AD pathophysiology. Furthermore, they represent important tools for the development of new strategies for the pharmacotherapy of AD and related neurodegenerative disorders.

Table 1. Some transgenic mouse lines developed for the purpose of modeling AD and their associated pathologies in relation to the human disease.

Mouse model	**Histopathology/behavioral impairments/synaptic plasticity**	**Reference**
Human APP695 mutant	Amyloid plaques/memory deficits/LTP deficits in hippocampal CA1 and dentate gyrus	Chapman et al., 1999; Hsiao et al., 1995; Hsiao et al., 1996
Human APP	Neuronal loss/spatial learning deficits/NMDA-dependent LTP deficits in CA1	Nalbantoglu et al., 1997
Human PS-1 mutant	Increase in Aβ 1-42/43 production/increased synaptic plasticity in CA1	Borchelt et al., 1997
APP695/PS-1	Aβ deposits in neocortex and hippocampus/memory deficits/accelerated decay of LTP	Borchelt et al., 1997; Puoliväli et al., 2002
APPswe/PS1dE9	Aβ plaque deposition, episodic-like memory impairments, somatostatin level deficit in neocortex, deficits in cholinergic markers in neocortex and hippocampus	Savonenko et al., 2005
APPswe/PS1M146V/tauP301L	Deposition of Aβ plaques, neurofibrillary tangles in the neocortex and hippocampus	Oddo et al., 2003

APP-amyloid precursor protein; PS-presenilin; LTP-long-term potentiation; NMDA-N-methyl-D-aspartate; Aβ-amyloid-β.

2.3 Estrogens and estrogen receptors

A large portion of the basal forebrain cholinergic neurons contain ERs. This gives an anatomical basis for estrogen actions on cholinergic neurotransmission.

2.3.1 Estrogens

Estrogens belong to a large family of hormones that are composed of three 6-carbon rings and one 5-carbon ring and are collectively called steroids (Kawata, 1995). The common steroid precursor is cholesterol. The latter is synthesized from acetate in all-steroid producing organs except the placenta. There are three forms of secreted estrogens: estradiol, estrone and estriol (Kawata, 1995). The most potent of estrogens is estradiol, whose potency is 12 times higher than that of estrone and 80 times that of estriol (Kawata, 1995). However, all of these hormones have a common precursor androgen. In the non-pregnant female, the majority of circulating estrogens is secreted by the ovaries. During pregnancy, the placenta becomes the main source of estrogenic hormones. In males, estradiol is synthesized from testosterone, but in a quantitatively lower amount (Speroff et al., 1982).

The main function of estrogens in the peripheral tissues of females is to trigger cellular proliferation and growth of the tissues related to reproduction, e.g. in sex organs. Most functions of estrogens are exerted through ERs that function as ligand-dependent transcription factors (Nilsson et al., 2001).

2.3.2 Estrogen receptors and their actions via genomic and non-genomic pathways

The receptors for estrogen are members of a large family of transcription factors, which also includes receptors for other steroids such as thyroid hormone and dihydroxyvitamin D_3 (Bloom and Kupfer, 1995). To date, two types of ERs are known: ERα and ERβ (Nilsson et al., 2001). As with most other transcription factors of this class, both ERs contain a highly conserved DNA binding domain, consisting of two Zinc finger protein motifs. However, both receptors display significant differences in their C-terminal, which contains the ligand binding domain (in rat 58% amino acid homology). At the N-terminal domain, there is no homology between the receptors at all (Toran-Allerand, 1996). Functionally, the ligand binding domain is the most active site in the ER structure (Hermanson et al., 2002). It is involved both in high affinity ligand binding and receptor dimerization (Littleton-Kearney et al., 2002). ERs are encoded by different genes, located

on chromosome 6 (ERα) and chromosome 14 (ERβ) (Hall et al., 2001). Furthermore, their binding affinities, ligand specificities as well as tissue distribution are different (Toran-Allerand et al., 1999). Recently, evidence for the existence of a possibly novel, plasma membrane-associated ER-X was reported (Toran-Allerand et al., 2002). However, ER-X shares some homology with the C-terminal of ERα (Toran-Allerand, 2004). Therefore, further studies are needed to reveal whether ER-X is an unknown form of ERα or is entirely novel in origin.

ERs act directly as ligand-dependent transcription factors. According to the classical concept of steroid action (see Figure 3), under normal conditions some steroid receptors such as the ERs shuttle between cytoplasm and nucleus (Ylikomi et al., 1998). In the cytoplasm, ERs are associated with a variety of proteins such as the heat shock protein 90 kDa (Hsp90) which has been shown to be responsible for the inhibition of ER DNA binding (Ylikomi et al., 1998). In the presence of a receptor-activating ligand, the ER-Hsp90 complex dissociates, which results in the nuclear translocation of the ligand-carrying ER and, ultimately, binding to DNA. In addition to this classical pathway that is also called genomic, it has been claimed that ERs can be involved in gene transcription via various other signaling cascades in the cytoplasm or non-genomic signaling. Such cascades include mitogen activated protein kinase-, phosphatidylinositol 3 kinase-, cAMP response element binding proteins- (Behl, 2002) and protein kinase B-signaling pathways (Znamensky et al., 2003). In addition, in endothelial cells ER-mediated estrogen-dependent pathway affects cellular membranes in a way that leads to the activation of ras, raf, mitogen activated protein kinase kinase and the induction of the cell proliferation (Nilsson et al., 2001). The non-genomic ER-signaling pathway has been suggested to occur both with and without presence of the ligand. In case when the ligand is missing, ER-signaling pathway may function as a cross-talk between other signaling pathways (Hermanson et al., 2002).

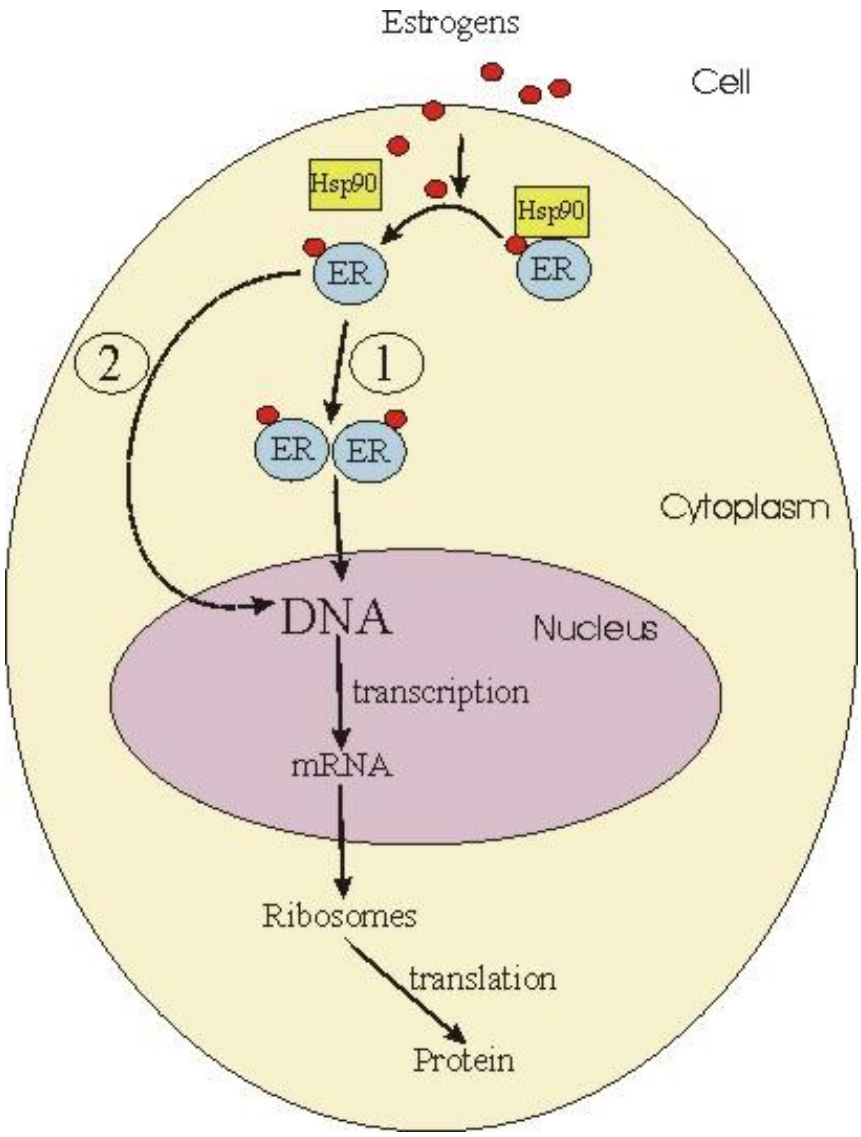


Figure 3. A simplified schematic diagram of intracellular action of estrogens via estrogen receptor (ER). Estrogens diffuse across the cell membrane and binds ER-heat shock protein 90 kDa (Hsp90) complex. (1) Genomic (classical) signaling pathway. Ligand binding causes conformational changes of receptors, release of chaperones, dimerization of the receptors and translocation directly to the nucleus. (2) Non-genomic signaling pathway. After binding of estrogens by ER, rapid induction of cAMP and Ca^{2+} release through second messenger systems or activation of mitogen-activated protein kinases, phosphatidylinositol 3-kinase and protein kinase B takes place in the cytoplasm. Gene activation in this case is affected by other transcription factors.

2.3.3 ERα and ERβ distribution in the central nervous system

In situ hybridization histochemistry in the rat central nervous system indicated the presence of both ERα and ERβ messenger RNAs (mRNA) through the rostral-caudal extent of the brain and spinal cord (Shughrue et al., 1997). Some brain regions contain mRNA of both receptors, whereas others exhibit ERα or ERβ mRNAs exclusively. For example, only ERα mRNA hybridization signal is detected in the ventromedial hypothalamic nucleus and subfornical organ. In contrast, only ERβ mRNA is observed in the neurons of the olfactory bulb, supraoptic, paraventricular, suprachiasmatic, and tuberal hypothalamic nuclei, zona incerta, ventral tegmental area, cerebellum (Purkinje cells), laminae III-V, VIII, and IX of the spinal cord, and pineal gland (Shughrue et al., 1997). Other brain regions such as the hippocampus, the amygdala and the cerebral cortex express both ERα and ERβ mRNA.

2.3.4 Effects of estrogens: lessons from experimental and population-based studies

The traditional site for the study of ovarian steroids' actions and their receptors was the hypothalamus, because of its control of reproductive function. More detailed ERs mapping studies revealed the distribution of the ERs in such regions as amygdala, hippocampus, neocortex, and cerebellum (Shughrue et al., 1997). A wide distribution of the ERs in the nervous system suggests that estrogen may be involved in a variety of physiological functions in neuronal cells. Indeed, many estrogen-dependent alterations have been described. These include the induction of ChAT in the basal forebrain (Gibbs et al., 1994), increases in the expression of tryptophan hydroxylase, which is the key enzyme in serotonin biosynthesis, the suppression of the serotonin transporter expression in the macaque raphe nuclei (Pecins-Thompson et al., 1996; Pecins-Thompson et al., 1998), time-dependent effects on the level of tyrosine hydroxylase mRNA in the catecholaminergic cells of brainstem (Liaw et al., 1992), heterogeneous effects on the dopamine turnover (increases in dorsomedial nucleus and decreases in rostral perivetricular, medial preoptic, and preoptico-suprachiasmatic nuclei) (Lookingland and Moore, 1984). Taken together, these observations show the complexity of estrogens' actions in the brain.

Besides their physiological actions, estrogens are also known to influence the morphology of neurons. For example, estradiol mediates hippocampal synapse density during the estrous cycle in rats (Woolley and McEwen, 1992; Woolley and McEwen, 1993). Rune et al. (2002) suggested that estradiol-induced spine formation on CA1 pyramidal cells may be mediated

presynaptically by activating ERα in CA3 pyramidal cells. Moreover, a number of studies revealed an improvement in learning and memory performance in ovariectomized (OVX) rodents (McEwen and Alves, 1999). However, data from the human population-based studies on the effect of ERT on cognitive functions in postmenopausal women were contradictory. Some studies reported that ERT is associated with better performance in visual and verbal memory tests, fine motor skills and somewhat poorer performance on tests of spatial recognition (Barrett-Connor and Kritz-Silverstein, 1993; Henderson et al., 1996; Kampen and Sherwin, 1994; Kawas et al., 1997). Paganini-Hill and Henderson (1994, 1996) reported that the risk of AD and other dementias might be significantly lower in ERT users, also with higher ERT doses and duration than in non-users. At the same time, no association between estrogen use and AD was found in a number of other studies (for review see Hogervorst et al., 2002). Studies that reported positive or negative findings were difficult to compare because of discrepancies in the methods used. For example, common selection bias when study subjects are from the health maintenance organisation (Brenner et al., 1994), unknown additional treatment or use of vaginal medication (Brenner et al., 1994; Henderson et al., 1996), a bias that is associated with patient compliance (Paganini-Hill and Henderson, 1994, 1996) or an additional hormone treatment (such as thyroid replacement therapy) could have influenced the reported results. Thus, large scale double blinded, placebo-controlled studies were needed to confirm the beneficial effects of estrogenic treatment on central nervous system.

The Womens' Health Initiative (WHI) study was a large-scale randomized clinical trial whose estrogen and progestin arm was prematurely stopped. The findings from this study showed that the benefit of taking ERT was outweighed by the increased risk of venous thromboembolism, stroke, and myocardial infarction in postmenopausal women (Rossouw et al., 2002). The disappointing results lead to the conclusion that ERT or HRT should not be recommended to postmenopausal women.

3. Aims of the study

The present work was designed to investigate the effects of estrogen deprivation and ERT on the number of cholinergic cells in the basal forebrain and their content of ERα in rodents.

The specific aims of this work were:

- To combine and apply stereological methods and improvements in the histological techniques to estimate the total number of cholinergic cells in the MSVDB, HDB and NbM (study I)
- To reveal the effect of ERT on the total number of cholinergic cells in the basal forebrain and their content of ERα in aged mice (study II)
- To investigate whether OVX and ERT affects hippocampal Aβ deposition load in double transgenic (APP/PS1) mice carrying mutated amyloid precursor protein (APPswe) and presenilin-1 (PS1-A246E) (study III).
- To estimate the cholinergic cells and their content of ERα in APP/PS1 transgenic mice and their wild-type littermates at 6 and 12 months of age (study IV)

4. Materials and methods

4.1 Animals

Animal species, strains, age and number of animals used in this series of studies are presented in Table 2. The animals were singly housed in a controlled environment (National Animal Center, Kuopio, Finland; temperature 22°C, humidity 50-60%, lights on from 0700 to 1900 hours) with water and food freely available. In study II, C57BL/6J mice with some genetic background derived from 129/Sv and DBA/2J inbred strains were used. In the studies III and IV, transgenic mice expressing either human PS1 harboring the familial AD-linked A246E mutation (strain background = C3H/HeJ × C57BL/6J F3) or chimeric mouse/human APP695 harboring a human Aβ domain and mutations (K595N, M596L) linked to Swedish familial AD pedigrees (strain background = [C3H/HeJ × C57BL/6J F3] × C57BL/6J n1) were backcrossed to C57BL/6J mice for 6 generations. Subsequently, these lines were crossed together to generate double transgenic mice coexpressing both transgenes (Borchelt et al., 1997).

Table 2. Characterization of the animals and histology used in the studies.

Study	Animal species	Strain	Animal number	Animal age, months	Histology/immunohistochemistry
I	Rat	Wistar	4	3	ChAT and ERα immunolabeling
II	Mouse	C57BL/6J*	20	21	ChAT and ERα immunolabeling
III	Mouse	APP/PS1	75	9 and 17	Modified Bielschowsky's silver staining
IV	Mouse	APP/PS1	57	6 and 12	ChAT and ERα immunolabeling

* C57BL/6J mice with a small contribution from 129/Sv and DBA/2J strains; APP/PS1-double transgenic mice carrying mutated amyloid precursor protein (APPswe) and presenilin-1 (PS1-A246E); ChAT-choline acetyltransferase; ERα-estrogen receptor alpha.

All experiments were permitted by the National Laboratory Animal Center and were done according to the guidelines set by the Council of Europe and the State Provincial Office of Eastern Finland.

4.2 Ovariectomy

The mice were anesthetized with a mixture (8 ml/kg of body weight, i.p.) of sodium pentobarbital (46 mg/kg; Synopharm, Germany) and chloral hydrate (47 mg/kg; Merck, USA). The fur on the both sides of body was shaved from hip to the lowest rib, bilateral incisions were made and the ovaries and surrounding tissue were removed. The incision was closed by suturing the muscles and stapling the skin. In the sham-operated (SHAM) group of mice, only skin and muscles were cut but the ovaries were not removed.

4.3 17β-estradiol treatment

Each animal from the OVX (studies II, III and IV) or SHAM (Study III) groups treated with 17β-estradiol (OVX+E and SHAM+E, respectively) had a subcutaneously implanted estrogen pellet containing 0.18 mg of 17β-estradiol (Innovative Research of America, USA) that delivers a continuous supply of estrogen for 90 days. These pellets yield serum estradiol levels of 50-75 pg/ml, which is similar to the serum estradiol levels of 35-75 pg/ml reported in mice during proestrus (Grasso and Reichert, 1996; Nelson et al., 1992).

4.4 Histology

The mice were deeply anaesthetized with a mixture (8 ml/kg of body weight, i.p.) of sodium pentobarbiturate (46 mg/kg; Synopharm) and chloral hydrate (47 mg/kg; Merck). Thereafter they were perfused through the heart, first with saline (3 min), then with a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.26% picric acid in 0.1 M phosphate buffer (PB), pH 7.4. The animals were coded so that the experimenters did not know what treatment they had received during the study. Brains were removed from the skulls and 40 µm-thick sections were cut on a Leica VT 1000 S vibratome into 4 (studies II and IV) or 6 (study I) series. One series from each animal was randomly selected and further processed for immunohistochemistry.

4.4.1 Immunohistochemistry

The sections were extensively washed in PB and immersed in a mixture of 25% sucrose and 10% glycerol in 0.05 M PB, and freeze-thawed in liquid nitrogen in order to increase the penetration of antisera during immunostaining. Next, sections were washed with 0.05 M Tris buffered saline, pH 7.4 (TBS), 2 times for 20 min, and with 0.5% Triton X-100 TBS for 15 min. Non-specific binding sites for subsequently applied immunoreagents were then blocked with 10% normal goat serum (NGS; Colorado Serum Company, USA) for 40 min, followed by the treatment of sections with 1% NGS in TBS for 10 min. The sections were incubated for 48 hours at 4°C in a polyclonal rabbit anti-ERα antibody (1:10000, Santa Cruz Biotechnology, USA, catalog no. sc-542; Pavao and Traish, 2001) that recognizes the C-terminal domain of the ERα. Extensively rinsing of sections was then followed by an incubation with biotinylated anti-rabbit IgG (1:300 Vector BA-1000, USA) overnight at 4°C and then in avidin/biotin horseradish peroxidase complex (ABC, 1:500 Vector PK-4000) for 3 hours at room temperature. The immunoperoxidase reaction was carried out using ammonium nickel sulfate-intensified 3,3′-diaminobenzidine (DAB) as a chromogen, giving a blue-to-black granular reaction product. After further extensive washing, the ERα stained sections were incubated in rabbit anti-ChAT antiserum (1:4000 Chemicon AB 143, USA, publications II and IV; Bruce et al., 1985) or monoclonal rat anti-ChAT antibody (1:10, 770990; Roche, Basel, Switzerland; study I; Eckenstein and Thoenen, 1982) for 48 hours at 4°C followed by incubation in goat anti-rabbit IgG (1:300 Jackson 111-005-003, USA; studies II and IV) or rabbit anti-rat IgG (1:50, AB-136; Chemicon; study I) for 6 hours at room temperature, and then in rabbit peroxidase anti-peroxidase complex (1:400 DAKO ZO 113, Denmark; studies II and IV) or rat peroxidase anti-peroxidase complex (1:300, PAP-20, Chemicon; study I) overnight at 4°C. The second peroxidase reaction was carried out using plain DAB as a chromogen, resulting in a homogeneous brown end product. For all washing steps 0.05 M TBS pH 7.4 containing 1% NGS served as dilutent for the antisera. Sections were washed 3 times for 30 min between the use of all immunoreagents.

Double immunoperoxidase labeling of ERα (Santa Cruz Biotechnology) and ChAT was also performed based on rat anti-ChAT (Boehringer Mannheim Biochemica, Germany) or goat anti-ChAT (Chemicon AB 144P, USA). Control stainings for immunohistochemistry were carried out by omission of one or both primary antibodies. No cellular staining was observed in these controls.

4.4.2 Modified Bielschowsky's silver staining

Bielschowsky's silver staining was performed according to a modification by Yamamoto and Hirano (1986). Tissue sections were placed in 20% silver nitrate for 20 min in the dark. Subsequently, sections were removed and placed into distilled water. Evaporated (for 20 min) ammonium hydroxide was added to the silver nitrate solution drop by drop, stirring vigorously until the precipitate turned clear. Then, 2 more drops of ammonium hydroxide were added. The sections were returned to this solution for 15 min in the dark. They were then transferred into ammoniacal distilled water (3 drops of ammonium hydroxide in 100 ml distilled water). Subsequently 3 drops of the developer (containing 20 ml formalin, 1 drop concentrated nitric acid and 0.5 g citric acid in 100 ml distilled water) were added to the solution of the ammoniacal silver nitrate. The sections were allowed to remain in this solution until the fibers are black with a tan background which was controlled under the microscope. The whole development procedure took 3-5 min. Then sections were washed in distilled water, dehydrated and mounted in Durcupan.

4.4.3 Tissue embedding

Durcupan. After thorough washing in TBS, free-floating sections were rinsed with distilled water, dehydrated in a series of ethanol (50%, 70%, 90%, 96% for 5 min in each and in absolute ethanol twice for 5 min) and propylene oxide twice for 5 min. The sections were then immersed in Durcupan (AMC, Fluka, Buchs, Switzerland). After 3 hours at room temperature in Durcupan, the sections were transferred onto slides and covered with a coverslip. To ensure that the sections were planar between the coverslip and the objective slide and that excess Durcupan was removed, a glass block (weight ~50 g) was placed on the coverslip to slightly press the coverslip. The Durcupan was subsequently polymerized at 60°C for 24 hours.

Depex. After thorough washing in TBS, the sections were mounted on gelatin-coated slides and dried overnight at 37°C. Thereafter, sections were dehydrated in absolute ethanol, cleared in xylene, embedded with Depex and coverslipped.

4.5 Stereology

In our experiments, the optical fractionator method (Gundersen, 1986) was used to estimate cell numbers. The analysis was done using StereoInvestigator software (MicroBrightField,

USA). For immunostaining, one series of samples in four (studies II and IV) and six (study I) was randomly selected using a random number table. The nuclei were first outlined using CFI Plan Achro 4× objective. Thereafter, a CFI Plan Fluor 100× oil immersion objective (N.A. 1.30, W.D. 0.20 mm, optical depth 0.16 μm) was used for the counting. The main parameters of the stereological counting were the following: a grid size-6400 μm^2, an area between each counting frame was 1225 μm^2, the mean thickness of the mounted section-30 μm, the guard zone was set to 5 μm from the surface of the section, and the disector height-20 μm. The counting criterion was that the top of the cell body comes into focus inside the disector height. The counted ChAT-ir neurons were divided into the following groups: 1) neurons that were positive for ChAT-ir only; and 2) neurons that were positive for both ChAT- and ERα-ir (ChAT/ERα-ir). The latter group in study IV was subdivided into ChAT-ir neurons that contained nuclear ERα-ir and neurons that contained cytoplasmic ERα-ir. The total number of ChAT-ir neurons in all studies was the sum of all counted cell groups.

4.6 Plaque counting

Systematic uniform sampling with random starting point was used to select every eighth section for modified Bielschowsky's silver staining (Yamamoto and Hirano, 1986). After the staining, each visualized plaque on each section was plotted using the StereoInvestigator program (MicroBrightfield Inc., USA). Counting was performed in the entire rostrocaudal extent of the hippocampal formation including the dentate gyrus, hippocampal proper, and subicular complex.

4.7 Statistical analysis

The statistical analyses were made using the SPSS for Windows program (v. 9.0 and 10.0; SPSS Inc., USA). One-way analysis of variance (ANOVA) followed by Bonferroni and Scheffe's post hoc tests, univariate ANOVA and *t* test (Altman, 1991) were used to analyze the treatment effects and the group and treatment interactions on different variables. The level of statistical significance for all values was set at $P<0.05$. The statistical methods are described in detail in publications I-IV.

5. Results

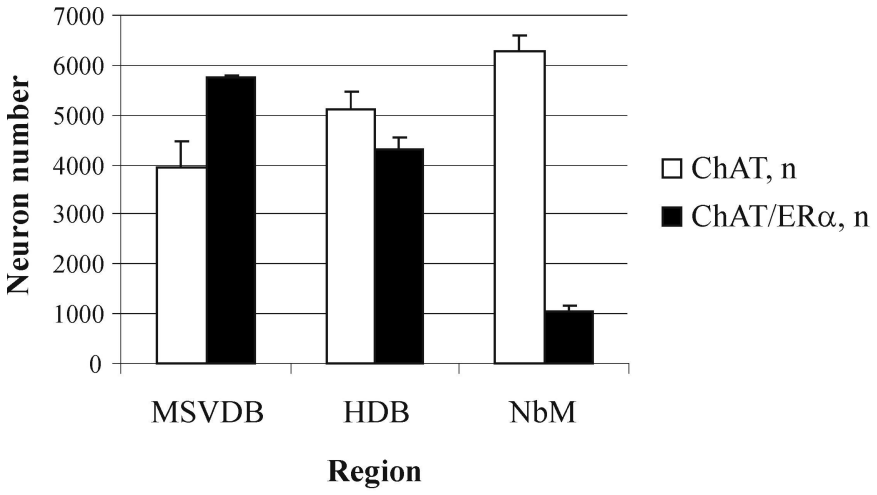
5.1 Durcupan embedding

The mounting in the epoxy-resin Durcupan was used in studies I-IV in order to maintain section thickness, while the routine mounting in Depex caused a flattening of sections. The clear difference in section thickness of Depex and Durcupan mounted material was revealed in study I. The thickness of mounted sections was 12.8±0.1 μm in Depex and 40.8±0.35 μm in Durcupan. Macroscopically, sections mounted in Durcupan with its intrinsic brownish color were darker than those in Depex. However, at the microscopic level both chromogens in double immunostained sections were clearly distinguishable. Furthermore, Durcupan embedding facilitated the identification of individual cells while focusing through the section.

5.2 The total number of cholinergic neurons in the basal forebrain of young rats

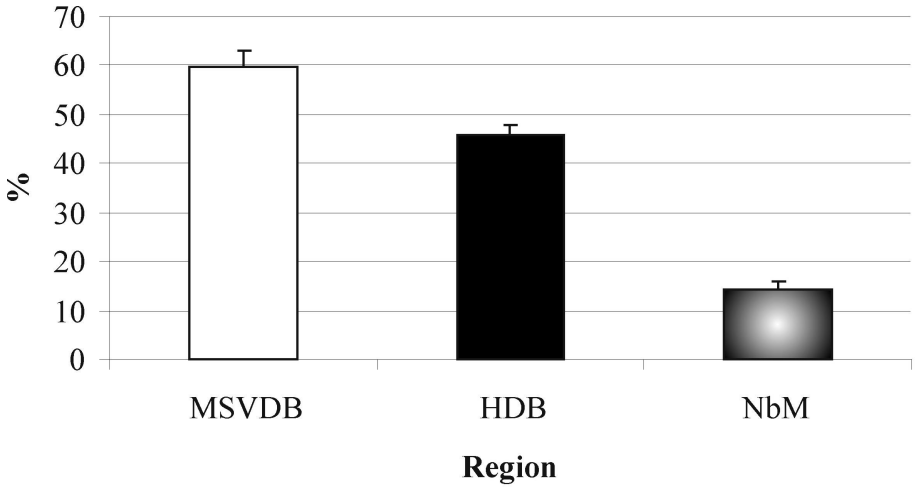
In study I, the total number of ChAT-ir neurons was estimated from the main cholinergic basal forebrain nuclei of male Wistar rats. Animals were sacrificed at the age of 3 months. The numbers of ChAT-ir and ChAT/ERα-ir neurons from each cholinergic nucleus are presented in Figure 4A. The highest percentage of ChAT-ir cells that contained ERα-ir was observed in the MSVDB (Figure 4B). The NbM had the lowest percentage of ChAT/ERα-ir from all analyzed regions.

Figure 4A. Numbers of ChAT- and ChAT/ERα-ir neurons in the rat basal forebrain.



MSVDB-medial septum-vertical diagonal band; HDB-horizontal diagonal band; NbM-nucleus basalis magnocellularis; ChAT-choline acetyltransferase; ChAT/ERα-choline acetyltransferase and ERα colocalized.

Figure 4B. Percentage of ChAT/ERα-ir neurons in the rat basal forebrain cholinergic nuclei.



MSVDB-medial septum-vertical diagonal band; HDB-horizontal diagonal band; NbM-nucleus basalis magnocellularis.

5.3 Amyloid plaque counts in transgenic mice

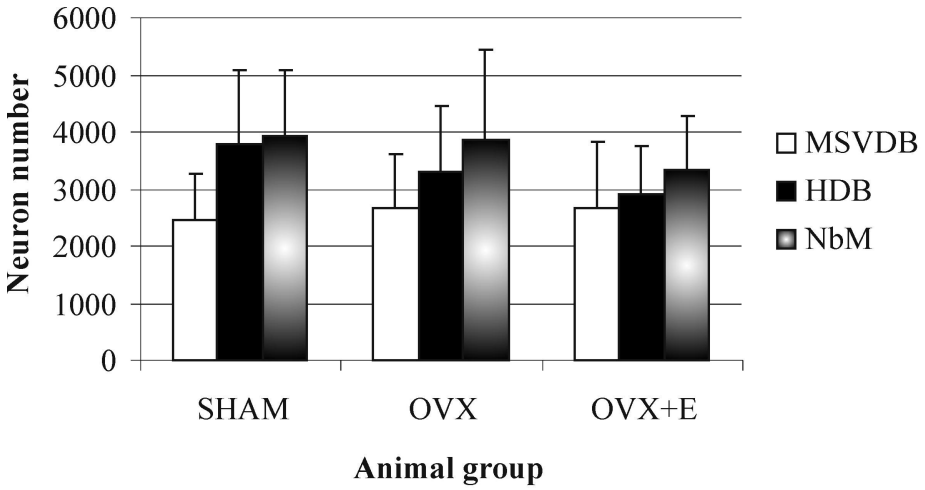
The modified Bielschowsky's silver staining revealed aggregates of argyrophilic material in plaque-like formations and neurotic profiles throughout the brain of APP/PS1 mice. The size and shape of the plaque-like formations were similar to those observed earlier using Aβ immunostaining (Borchelt et al., 1997). First plaques were detectable at the age of 9 months and were located in the region of the subiculum. At the age of 17 months, the deposits were also detected in the molecular layer of the dentate gyrus and the stratum lacunosum-moleculare of the CA2-3 subfields of hippocampus. In the CA1 subfield, plaques were scattered across different layers.

Seventeen months old APP/PS1 SHAM animals had approximately 15 times higher number of deposits than that in the 9-month-old mice (ANOVA, $P<0.001$). However, no statistically significant differences were observed in amyloid plaque counts between the groups (SHAM, OVX, and estrogen treatment) at the age of 9 ($F(2,24)=2.1$, $P=0.2$) or 17 ($F(2,49)=0.7$, $P=0.5$) months. Amyloid plaque counts between SHAM and SHAM + E mice at the age of 17 months did not differ (*t* test, $P=0.8$).

5.4 Effects of estrogen on the total number of cholinergic neurons and their content of ERα in aged mice

The total numbers of ChAT-ir neurons in the MSVDB, HDB and NbM of 21-month-old female mice were not influenced by OVX or 17β-estradiol treatment (Figure 5A).

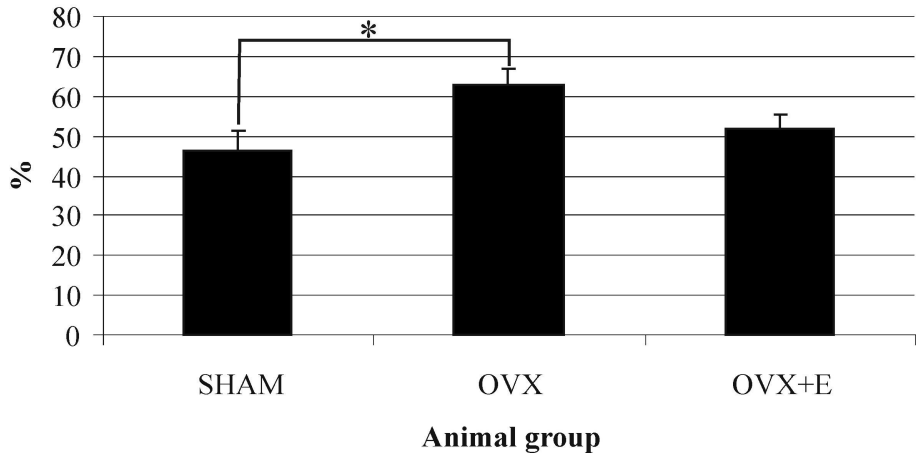
Figure 5A. Total numbers of ChAT-ir neurons in the MSVDB, HDB and NbM of different treatment groups.



SHAM-sham-operated; OVX-ovariectomized; OVX+E-ovariectomized and treated with 17β-estradiol; MSVDB-medial septum-vertical diagonal band; HDB-horizontal diagonal band; NbM-nucleus basalis magnocellularis.

The percentage of ChAT/ERα-ir cells in the MSVDB was significantly higher in the OVX group than that in SHAM mice (Figure 5B; ANOVA, Bonferroni post hoc test, P=0.036). Interestingly, such difference was not observed in other analyzed regions, i.e. the HDB and NbM.

Figure 5B. The percentage of ChAT/ERα-ir neurons in the MSVDB of different treatment groups.

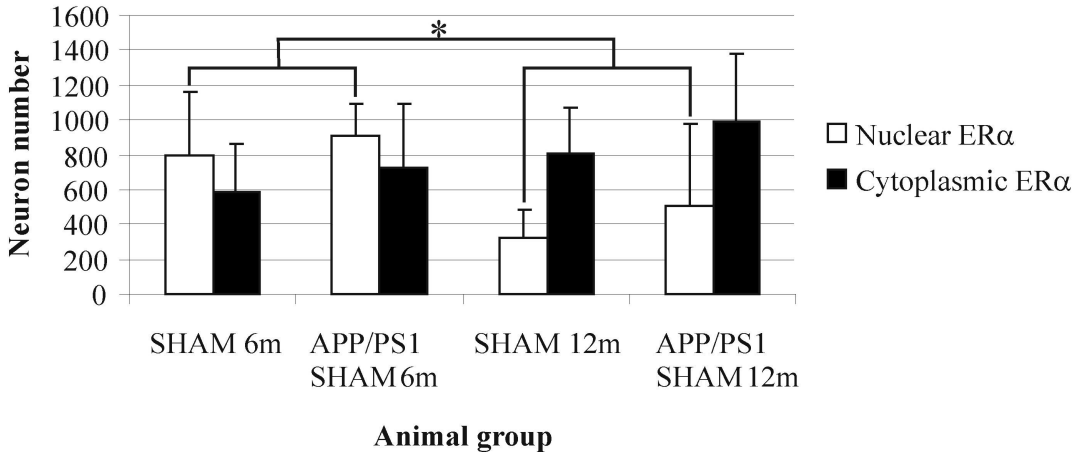


* ANOVA, Bonferroni post hoc test, *P*=0.036; SHAM-sham-operated; OVX-ovariectomized; OVX+E-ovariectomized and treated with 17β-estradiol.

5.5 Nuclear and cytoplasmic localization of ERα in cholinergic neurons

In study IV, the ChAT/ERα-ir neurons were subdivided into those having nuclear ERα-ir and those having cytoplasmic ERα-ir. The numbers of ChAT-ir neurons containing nuclear and cytoplasmic ERα-ir from the MSVDB of 6 and 12 month old mice are presented in Figure 6. There were no significant differences between the treatment groups or age groups in the total number of ChAT-ir neurons, number of ChAT/ERα-ir neurons or the percentage of ChAT/ERα-ir neurons. However, the number of ChAT-ir cells containing nuclear ERα-ir was significantly lower in 12-month-old than in 6-month-old mice (ANOVA, $P<0.001$).

Figure 6. The number of ChAT-ir neurons containing nuclear and cytoplasmic ERα-ir in the MSVDB of 6 and 12 months old mice.



* ANOVA, *P*<0.001; SHAM 6m-sham-operated, 6-month-old; APP/PS1 SHAM 6m-APP/PS1 double transgenic, sham-operated, 6-month-old; SHAM 12m-sham-operated, 12-month-old; APP/PS1 SHAM 12m-APP/PS1 double transgenic, sham-operated, 12-month-old.

6. Discussion

6.1 Experimental animals

Age of animals. Experiments in the present work were performed with 3-month-old rats and 6, 9, 12, 17 and 21 months old female mice that are representatives of different age groups according to the following age criteria: animals become adult at the age of 2-3 months, when rodents start to be sexually mature. At the age of 6-12 months mice would correspond to adulthood, whereas 17-21-month-old are mostly in a postmenopausal state.

Ovariectomy as a model in estrogen studies. OVX animals are widely used in experimental studies as controls for estrogen-replaced animals. The ovaries are the main source of systemic estrogen for non-pregnant adult females. However, other sites of estrogen biosynthesis are present throughout the body, e.g., in adipose tissue. After the ovaries are removed, animals gain weight (Gale and Sclafani, 1977). Therefore an increase in adipose tissue may result in greater local biosynthesis of estrogen catalyzed by aromatase, a terminal enzyme in local estrogen biosynthesis activity (Simpson et al., 1999). Davidge and colleagues (2001) studied whether OVX might be a confounding factor for estrogen-related experimental studies. Although the body weight in OVX Sprague-Dawley rats was greater than that in normally cycling animals, plasma 17β-estradiol levels were significantly higher in the cycling rats. The uterine weight which is a biological marker of estrogen level was statistically similar in cycling and OVX groups. However, from the vascular responses in mesenteric arteries the authors concluded that OVX-only model without a calorie-controlled diet may preclude an accurate determination of the effects of estrogen. In the current studies II-IV, OVX animals had clearly lower uterine weights than those in SHAM animals. This is indicative of the difference in the systemic estrogen level between these groups. Furthermore, in none of the studies (II-IV), statistically significant differences in body weight between the treatment groups were observed. Thus, greater local biosynthesis of estrogen is unlikely to confound the presented results.

Transgenic model for AD. Transgenic mice used in experiments III and IV carried mutated APPswe and PS1-A246E genes. This transgenic mouse strain exhibits clear neuropathological changes such as accumulation of amyloid plaques in the brain starting around the age of 9 months (Borchelt et al., 1997). The formation of amyloid plaques is primarily detectable in the subiculum and caudal cortex, and extends later to hippocampus and other cortical areas. In addition, at the age of 12 months, these transgenic mice exhibit significant deficits in water maze learning when compared to wild type littermates (Puoliväli et al., 2002). On the other hand, these

mice do not show cholinergic cell loss in the basal forebrain at the adult age. However, it is possible that visible pathological changes in the cholinergic nuclei of the basal forebrain show up later. Nevertheless, the relatively late age when these mice develop neuropathological and behavioral changes is advantageous in studies where different treatment strategies can be tested.

6.2 Methodological considerations

Embedding material. Durcupan embedding was routinely used to ensure an accurate estimation of the total numbers of ChAT- and ChAT/ERα-ir neurons. In general, the optical fractionator method (Gundersen, 1986) is designed to make estimations despite the shrinkage of the analyzed tissue. However, the shrinkage of the tissue can affect the analysis itself. Study I showed that sections which undergo the commonly used dehydration procedure and embedding in Depex mounting material shrink up to 74% of their original thickness. In contrast, sections embedded into Durcupan lose only 20% of their thickness. This residual thickness loss is probably due to chemical procedures sections undergo during immunostaining (e.g. Triton X-100 treatment) or mounting. Durcupan embedding also facilitated the recognition of distinct cells while scanning the sections and allowed a better verification of the optimal penetration of the antibodies throughout the section than in Depex-embedded material. Due to its advantageous properties compared with commonly used Depex, Durcupan was preferentially applied in the presented stereological studies.

Stereology. The term sterology has been introduced in the early 1960s by Hans Elias (see Mouton, 2002) and has its origin from the Greek word *stereo* to be translated as ‘solid’. As a method, stereology employs a three-dimensional analysis of biological structures. The important milestones of the modern stereological methods are unbiased sampling and the estimation of such parameters as the cell number, area, volume and length. This unbiased estimation introduces clarity. Furthermore, the comparison of results from different experiments executed by different research groups is reliable. In this series of studies, one of stereological methods, an optical fractionator (Gundersen, 1986), was used to count the total numbers of cholinergic neurons from different basal forebrain nuclei. Although almost the same optical fractionator strategy was used in all studies, the coefficient of variance (CV) or, in other words, the degree to which a set of data points varies, differed in different experiments. For example, in study I, the CV of the total number of ChAT-ir neurons in the MSVDB of rats was 0.10. In study II and IV, the CV of the total number of ChAT-ir cells differed from 0.32 (study II, SHAM group) to 0.49 (study IV, 12 months old APP/PS1 SHAM group) in the mouse MSVDB. Indeed, the CV values in mice studies are high. Several factors could influence that and explain higher animal variability in cell numbers. First of all, strain of animals. In

study II, C57BL/6J mice that had a small contribution from 129/Sv and DBA/2J strains were used. It could be viewed as a limitation in the study results interpretation. It has been observed that the number of cholinergic neurons in the MSVDB region is highly dependent on the mouse strain (Schwegler et al., 1996). Furthermore, such extrinsic factors as aging, surgical manipulations, treatment and pathology which are related to transgenic lines may contribute to the observed variation.

Antibody selection. The selection of the antibodies for the immunohistochemical stainings plays a vital role in the interpretation of the results of the study. The specificity of ChAT and ERα antibodies that were used in studies I-IV and their ability to react with rodent ChAT and ERα proteins in the brain were well documented in previous studies (Eckenstein and Thoenen, 1982; Harkany et al., 2002; Jeon et al., 1998; Pavao and Traish, 2001; Rossier, 1981). In studies II and IV, the antibodies against ERα and ChAT were raised in the same species-rabbit. This fact may raise some concerns about the results and their interpretation. However, as described in the Materials and methods of studies II and IV, both antigens were consecutively labeled and it should be emphasized that the black precipitate of nickel-DAB indicating ERα apparently covered all immunoreagents used for this staining. This masking obviously prevented any interference with the subsequent brown immunolabeling of ChAT with plain DAB as chromogen. It is noteworthy that the ERα-immunostained nuclei never turned brown during the second immunostaining and were clearly distinguished from the cytoplasmic ChAT immunoreactivity. Furthermore, the control stainings carried out by omitting one or both primary antibody from the double staining procedure resulted in no staining for the corresponding antigenic sites. In addition, when a single immunostaining was performed, ERα immunoreactivity was seen both in the nucleus and to certain extent also in the cytoplasm, whereas ChAT immunoreactivity was exclusively found in the cytoplasm. Nevertheless, a concomitant detection of ERα and ChAT is also possible based on other alternative antibody combinations, for example, using polyclonal rabbit anti-ERα (Pavao and Traish 2001) and polyclonal goat anti-ChAT (Aucoin et al., 2005; Brauer et al., 2000; Härtig et al., 2002).

6.3 Ovariectomy and estrogenic treatment effects on the cholinergic neurons

In study IV, the numbers of ChAT/ERα-ir neurons in the MSVDB were not influenced by estrogen-related treatment at 6 and 12 months of age in both transgenic and wild-type mice. Moreover, the percentage of ChAT/ERα-ir neurons was independent from estrogen status and did not differ in these mice. Evidence from earlier studies examining the relationship between the estrogen status and cholinergic system suggested that estrogen treatment causes an increase in the

cholinergic parameters such as ChAT activity (Gibbs and Aggarwal, 1998), potassium-evoked acetylcholine release and ChAT mRNA expression (Gibbs and Aggarwal, 1998). The relationship between estrogen status and number of cholinergic cells was also investigated. Miller et al. (1999) reported that estrogen increases the number of ChAT-ir neurons in the bed nucleus of the stria terminalis of 5-month-old C57BL/6J mice lacking estrogen. Gibbs (1998), on the other hand, found no significant changes in the number of ChAT-ir profiles/section in the MS and NbM of 16 and 19 months old Sprague-Dawley rats sacrificed 3 or 6 months following ovariectomy when compared to gonadally intact or estrogen treated animals. However, the number of ChAT-ir neurons in the rat basal forebrain may depend on the different estrogen doses and duration of treatment in these studies. It is possible that the administration of physiologically high doses of estradiol for 1 or 2 weeks resulted in a significant increase in the number of ChAT-ir cells in the MS and NbM of Sprague-Dawley rats (Gibbs, 1997). In the presented studies II and IV, the administration of estrogen at physiological doses lasting 3 months had no influence on the number of cholinergic cells in mice. In conclusion, the ovariectomy and estrogen treatment *per se* hardly influenced the cholinergic system. However, the duration of treatment and dose of applied estrogen could be key factors which affect cholinergic cell survival.

6.4 Estrogen dose and effect on the cholinergic cells of the basal forebrain

In experimental studies, the effects of estrogens were studied in a variety of nanomolar and micromolar concentrations (Lee and McEwen, 2001). It is known that low estrogen concentrations may enhance the amplitude of kainate-induced currents in CA1 (Gu et al., 1999) and inhibit calcium currents in striatal neurons (Mermelstein et al., 1999). High concentrations (2 μM) of 17β-estradiol show neuroprotective effects *in vitro* (Bishop and Simpkins, 1994). In the basal forebrain of adult rats, ChAT mRNA fluctuates across the estrous cycle in adult rats (Gibbs et al., 1994; Gibbs, 1996). A dose related increase in the number of ChAT-like immunoreactive cells was observed in the MS and NbM (Gibbs, 1997). However, these effects lasted only 1-2 weeks. In addition, injections of 17β-estradiol that produced very high estrogen levels in the blood (400-900 pg/ml) did not affect the level of ChAT activity. Similarly, administration of estradiol to ovariectomized female rats had rather modest effects on the ChAT activity in the basal forebrain nuclei, but showed a significant increase in their projection fields (Luine, 1985). In studies II, III and IV, the implanted estradiol pellets yielded serum estradiol levels of 50-75 pg/ml, which is similar to the serum estradiol levels of 35-75 pg/ml reported in mice during proestrus (Grasso and Reichert, 1996; Nelson et al., 1992). Nevertheless, none of those studies showed any treatment

effect on the ChAT-ir neuron number in the basal forebrain nuclei. In general, this finding is in agreement with previously published results where the long-term treatment with physiological doses of 17β-estradiol did not affect the number of ChAT-positive cells in rats (Gibbs, 1997). This suggests that both in rats and in mice the number of cholinergic neurons of the basal forebrain may be increased only by short-term estrogen treatment.

6.5 Cholinergic system in transgenic mice with age dependent β-amyloidosis

The cholinergic system undergoes severe degeneration in AD. In study IV, the number of ChAT-ir neurons in the MSVDB of 6 and 12 months old APP/PS1 mice was investigated. At 12 months this animal model for AD develops some pathological features resembling AD pathophysiology such as learning and memory deficits (Puoliväli et al., 2002) and plaque accumulation (Borchelt et al., 1997). However, both number and distribution of ChAT-ir neurons remained unaltered in 12-month-old APP/PS1 mice. This finding is in agreement with earlier published data from other AD transgenic mouse lines. Hernandez et al. (2001) examined the number of ChAT-positive neurons in NbM/substantia innominata (NbM/SI) in 12 months old PS1-1M164V and APPTg2576 double mutant transgenic mice and concluded that the number of cholinergic neurons in the NbM/SI complex is the same as in non-transgenic littermates. Furthermore, total cholinergic innervation in the frontal cortex of APP/PS1 mice was essentially equivalent to the non-transgenic littermates at 3, 8, 12 and 18 months of age (Hernandez et al., 2001). Applying stereological methods, Jaffar et al. (2001) counted basal forebrain cholinergic neurons bearing the low-affinity p75 neurotrophin receptor in 12 months old APPswe/PS1M146L double mutant mice and found no significant difference with their littermate controls. All this evidence indicates that age dependent β-amyloidogenesis does not affect cholinergic neurons directly or is unable to cause cholinergic deterioration in mice.

6.6 Estrogen status modulation and ERα content in cholinergic cells in mice

It was suggested that estrogens together with other steroid hormones may act not only through the well-known genomic pathway, but could be also involved in the non-genomic activation of the cellular mechanisms (McEwen and Alves, 1999). The findings from study IV, where the number of ChAT-ir neurons containing nuclear ERα is significantly lower at 12 months of age than that at 6 months, suggest that the balance between genomic and non-genomic pathways

may be changed due to aging *per se*. Furthermore, a similar pattern of nuclear *versus* cytoplasmic distribution of ERs in cholinergic cells of MSVDB might be also present in older animals. Indeed, our own unpublished observations suggest that the number of ChAT-ir cells containing nuclear ERα-ir at 12 months and 21 months of age remain similar. Thus, changes that facilitate or disrupt ERα translocation from the cytoplasm to nucleus might occur in adult animals and remain irreversible later on. This would also mean that these changes are independent from the level of estrogenic hormones which is in accordance with the data on 21 months old mice. Interestingly, SHAM, OVX and OVX+E groups did not significantly differ in the number of ChAT-ir neurons containing nuclear ERα-ir. Whether higher proportion of the cytoplasmic ERα in cells is favoring non-genomic pathways requires further investigation.

Taken together, evidence of the redistribution of the ERα between nucleus and cytoplasm inside cholinergic neurons requires further studies that could explain the physiological meaning of such process.

6.7 Estrogen modulation and β-amyloid accumulation

The accumulation of amyloid peptides that frequently comprise 40-42 amino acids and are derived from APP is believed to play a major role in the etiology of AD (for review see Selkoe, 1991). Xu and colleagues (1998) demonstrated *in vitro* that physiological concentrations of 17β-estradiol cause a decrease of amyloidogenic Aβ forms and an increase in soluble form of Aβ in a dose-dependent manner. It was hypothesized that 17β-estradiol may increase a release of APP from the trans-Golgi network, eventually reducing the local concentration of APP available as a source for Aβ production (Xu et al., 1998). Subsequently, several research groups showed that OVX is associated with an increase in total Aβ levels as compared to intact controls (Petanceska et al., 2000; Zheng et al., 2002). This effect could be reversed through administration of estradiol. However, in study III, the hippocampal accumulation of Aβ was not influenced by OVX or 17β-estradiol treatment in OVX and SHAM-operated transgenic mice. These data are in contrast with some of the previous studies. It is possible that the duration of the OVX, the age when OVX was performed and the age of transgenic animals when first Aβ deposits occur play a crucial role. Based on the previous studies, long term (e.g. 3 months) OVX, which is performed before the age when animals develop Aβ deposits may affect the total amount of Aβ in the brain. Otherwise, estrogen modulation most likely has no influence on the accumulation, aggregation and deposition of Aβ in

susceptible brain regions. However, *post mortem* analysis of AD patients that have participated in ERT clinical trials might provide further insights.

6.8 General discussion

A main result of the present work was the finding from study IV that in older animals the number of cholinergic neurons of the MSVDB containing cytoplasmic ERα-ir increased when compared to younger mice. These changes could be triggered by factors that inhibit nuclear receptor shuttling through nuclear pores. This suggests that the intensity of the non-genomic pathway through which estrogens exert their functions is higher in older mice. Whether the disturbance of these pathways in older animals would lead to morphological or functional changes in the brain is unknown. It is also unknown whether the same age-related shift in subcellular ER distribution occurs in humans. However, if the redistribution of ERs in humans occurs as in mice, it may at least partially contribute to the negative outcomes of the WHI study (Shumaker et al., 2004). Although the number of women with dementia was small, this study revealed an increased risk of all types of dementia due to combined hormonal therapy. The treatment administered in that study contained conjugated estrogen and a derivative of progesterone, which was added to prevent cancer. However, progesterone limits tissue response to estrogen by decreasing the concentration of cytoplasmic ERs (Speroff et al., 1982). In other words, progesterone suppresses estrogens' actions through the non-genomic pathway whose intensity and, possibly, importance increases during aging. Then, the administration of progesterone derivatives could mask beneficial actions of estrogens' systemically by decreasing the amount of cytoplamic ERs.

The negative findings from WHI study urged on a search for new therapeutic strategies that could be useful to prevent dementia and AD. Recently, a randomized, placebo-controlled study The Multiple Outcomes of Raloxifene Evaluation study provided interesting data (Yaffe et al., 2005). The use of raloxifene, a selective ERs modulator (SERM), resulted in reduced risk of cognitive impairment by 33% in postmenopausal women. Furthermore, taken into account that different ER polymorphisms could have different association with cognitive impairment (Pirskanen et al., 2005; Yaffe et al., 2002), the development of more specific SERMs could result in even greater reduction of people at risk. The combination therapy of ERT and cholinesterase inhibitor tacrine caused a cognitive improvement in AD cases (Schneider and Farlow, 1997). Therefore, if estrogens can be replaced with safer and more potent SERMs, the development of selective cholinergic drugs would become desirable. In AD, the main cholinergic nuclei that show degeneration are located in the basal forebrain. Thus, the combination of the cholinesterase inhibitors that would selectively target basal forebrain cells with SERMs may result in a significant

step towards delaying the progress of the disease. Future experimental and population-based studies should critically evaluate this possibility.

7. Conclusions

This series of studies was focussed on changes in the total number of ChAT-ir neurons in the basal forebrain, their content of ERα-ir, and Aβ accumulation in the brain in response to estrogen modulation. The results show that the long-term modulation of estrogen status may influence the intracellular content of ERα in the cholinergic neurons, but is unable to affect the number of cholinergic cells or load of Aβ in the brain. Unexpectedly, the intracellular localization of ERs seems to be independent from estrogenic treatment and AD transgenic phenotype, but altered by age *per se*.

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APPENDIX: ORIGINAL PUBLICATIONS (I-IV)

I

Estimation of the total number of cholinergic neurons containing estrogen receptor-α in the rat basal forebrain

Miettinen RA, Kalesnykas G, Koivisto EH.

Journal of Histochemistry & Cytochemistry 2002, 50(7):891-902.

II

Cholinergic neurons in the basal forebrain of aged female mice

Kalesnykas G, Puoliväli J, Sirviö J, Miettinen R.

Brain Research 2004, 1022(1-2):148-156.

III

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, and Puoliväli J.

Experimental Neurology 2004, 187(1):105-117.

IV

The effect of aging on the subcellular distribution of estrogen receptor-alpha in the cholinergic neurons of transgenic and wild-type mice

Kalesnykas G, Roschier U, Puoliväli J, Wang J, Miettinen R.

European Journal of Neuroscience 2005, 21(5):1437-1442.

PUBLICATIONS

SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

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