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Galanin and NPY in the rodent brain: rapid effects of 17β-estradiol and possible roles in hippocampal plasticity

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The **cover illustration** depicts the overall structure of the hippocampal formation including cornu ammonis (CA) and the dentate gyrus (DG). 17β-estradiol depicted as a chemical structure, galanin and neuropeptide Y may influence the plasticity of the hippocampal formation.

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"Patience tastes sour,
but gives the sweetest fruits"

To:
Jörgen, Giovanna, Rebecca,
Michaela, Max, Josefine
& Emilia, with endless love

Abstract

The neuropeptides galanin and neuropeptide Y (NPY) play an important role in the reproduction of rodents, e.g. by modulating the release of gonadal hormones, the nutritional status by effects on feeding behavior and also by influencing mating behavior. There are age- and gender- differences in galanin- and NPY like immunoreactivities (LIs) in brain areas important for higher functions, including the hippocampal formation (HiFo) and cortex, effects that are related to the concentrations of 17β-estradiol.

Neuropeptides in general are currently not considered critical in normal intergrative neuronal functions but are rather thought to act as slow modulators during periods of stress or injury. In the present thesis we attempted to investigate, if the normal cyclical changes in the female sex-hormone 17β-estradiol can affect neurotransmission in brain areas important for memory, cognition and mood. We studied not only ”long term” (days and weeks) - but also ”short-term” (one hour) - effects on galanin and NPY concentration in 17β-estradiol-primed ovariectomized rats and mice.

Radioimmunoassay (RIA) of galanin-like immunoractivity (LI) in extracts of brain tissues from ”long-term” 17β-estradiol-treated ovx rats showed that its effects on galanin is dependent on both dose and on duration. Galanin- and NPY-LI in brain tissues of young ovx rats and mice increased in response to 17β-estradiol treatment in the HiFo, frontal cortex and striatum already within an hour. This effect was not blocked by Tamoxifen®. Species differences were observed with regard to galanin, possibly due to tissue and species differences in the distribution of estrogen receptors. The mechanism(s) underlying the 17β-estradiol effects on galanin levels in the HiFo may be decreased release of galanin to the extracellular fluid since galanin-LI decreased in microdialysis samples two hours after a single injection of 17β-estradiol.

In the HiFo and caudate nucleus of mice, we found an increase in NPYtranscript after two hours by means of *in-situ* hybridization, perhaps a compensatory up-regulation of NPY mRNA after increased 17β-estradiol- induced release in these areas. Taken together with no effects of Tamoxifen® on the levels on galanin in the HiFo of rats, the short duration, and the fact that the density of classical estrogen receptors (ERs) seems to be limited in the striatum, we suggest that these effects are mediated through a membrane-related mechanism, perhaps not involving the classical ER route.

With an antiserum raised against the C-terminal end of the first 16 aminoacids of galanin - the sequence important for binding of galanin(1-29) to its receptor - we found evidence for a novel compound which appears to be a homologue to galanin. Chromatographical analysis revealed that it was not galanin(1-29) or the galanin related peptide GALP, but was with immunohistochemistry localised in the galanin systems in the brain and was further influenced by 17β-estradiol in the HiFo and frontal cortex in a similar manner as galanin(1-29).

Tissue concentrations of galanin, a putative galanin homologue and NPY can be altered within one hour by 17β-estradiol treatment i.a. in the HiFo. These ”short-term” effects are most likely to be due to effects on estrogen-primed peptide release which might influence mechanisms important for memory, cognition and mood.

List of the papers

1. Hilke, S. Theodorsson, A. Fetissov, S. Åman, K. Holm, L. Hökfelt, T. Theodorsson, E. **Estrogen induces a rapid increase in galanin levels in female rat hippocampal formation - possibly a nongenomic/indirect effect.** European Journal of Neuroscience 2005;21:2089-2099

2. Hilke, S. Theodorsson, A. Rugarn, O. Hökfelt, T. Theodorsson, E. **Galanin in the hippocampal formation of female rats – effects of 17β-estradiol.** Neuropeptides 2005;39:253-257.

3. Hilke, S. Åman, K. Hökfelt, T. Theodorsson, E. **Rapid versus prolonged treatment with 17β-estradiol induces different effects on galanin and neuropetide Y concentrations in the brain of ovariectomized mice.** Submitted for publication.

4. Hilke, S. Hökfelt, T. Theodorsson, E. **A short estrogen-responsive N-terminal galanin homologue found in rat brain and gut with antiserum raised against rat galanin(1-16).** Neurochemistry Research, In Press.

Related papers

Kokaia, M. Holmberg, K. Nanobashvili, A. Xu, Z-Q D. Kokaia, Z. Lendahl, U. Hilke, S. Theodorsson, E. Kahl, U. Bartfai, T. Lindvall, O. Hökfelt, T. **Suppressed kindling epileptogenesis in mice with ectopic overexpression of galanin.** Proceedings of the National Academy of Science 2001;98:14006-14011.

Holmberg, K. Kuteeva, E. Brumovsky, P. Kahl, U. Karlström, H. Lucas, GA. Rodriguez, J. Westerblad, H. Hilke, S. Theodorsson, E. Berge, OG. Lendahl , U. Bartfai, T. Hökfelt, T. **Generation and phenotypic characterization of a galanin overexpressing mouse.** Neuroscience. 2005;133:59-77.

Contents

Abbreviations

5HT	5-Hydroxytryptamine = serotonin
ACh	Acetylcholine
AD	Alzheimer´s disease
AP 1	Activator protein 1
BSA	Bovine serum albumin
CA	Cornu Ammonis
cAMP	cyclic adeno monophosphate
CAPS	Calcium- dependent activator protein for secretion
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CNS	Central nervous system
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CRH	Corticotropin-releasing hormone
DAG	Diacylglycerol
DG	Dentate Gyrus
DRN	Dorsal raphe nucleus
ECF	Extracellular fluid
EGTA	ethylele glycol-bis(β-aminoethylether)-n,n,n´,n´-tetraacetic acid
ER	Estrogen receptors
ERE	Estrogen-responsive elements
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
DBH	Dopamine beta-hydroxylase
DG	Dentate gyrus
FSH	Follicle-stimulating hormone
GABA	Gamma - amino butyric acid
GALP	Galanin-like peptide
GalR	Galanin receptor
G_i	G protein (inhibitory)
GnRH	Gonadotrophin-releasing hormone
G_p	G protein (activation of phospholipase C)
G_s	G protein (stimulatory)
HiFo	Hippocampal formation
HPA	Hypothalamus-pituitary-adrenal
HPLC	High pressure liquid chromatography
^{125}I	Radioactive iodine
IP_3	Inositol-1,4,5- triphosphate
LC	Locus Coeruleus
LDCV	Large dense core vesicles
LH	Lutenizing hormone
LI	Like immunoreactivity
LTP	Long term potentiation (of neurons)
MAPK	Mitogen activated protein kinase
mCi	Millicurie
MEK	Protein kinase that phosphorylates the ERK gene product
NA	Noradrenaline

NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
ovx	Ovariectomized
OVX	Ovariectomy
PYY	Neuropeptide tyrosine, tyrosine
Raf	Protein kinase
Ras	Small monomeric G proteins
ROD	Relative optical density
SSV	Small synaptic vesicles
Y_1	Neuropeptide Y, receptor 1
Y_2	Neuropeptide Y, receptor 2
Y_3	Neuropeptide Y, receptor 3
Y_4	Neuropeptide Y, receptor 4

Introduction

Steroid hormones in general and sexual hormones in particular play a role in a large number of crucial bodily functions utilizing a multitude of sophisticated signalling systems (see Knobil et al., 1994). The internal sexual hormone environment in females changes both during the entire life cycle (menarche, fertile age and menopause) and monthly during the estrous cycle of fertile age. The variation is particularly evident in the concentrations of 17β-estradiol (see de Vries 2004). Thus, in contrast to fertile males, the females 'normal basal conditions' mean continuously, over decades, fluctuating concentrations of sex hormones. Such fluctuations of 17β-estradiol are known to induce biological effects in the brain, in addition to their direct effects on the reproductive organs. The main purpose of the present thesis was to investigate the possible role played by the neuropeptides galanin and neuropeptide Y (NPY) for the biological effects of rapidly changing 17β-estradiol concentrations on the brain.

The most obvious role for estrogens in mammals is its negative and positive feedback action on the hypothalamic-pituitary axis to regulate the reproductive cycle (Knobil et al., 1994). The physiological meaning of the fluctuations in the plasma levels of estrogen is to create optimal conditions for reproduction, that is to induce ovulation and to prepare the uterus for implantation and growth of the fertilised egg, to control development and maintainance of the genitalia, as well as body habitus and the characteristics of sexual behaviour. Fertilization accomplishes the combination of the genes derived from the two parents and the creation of new organism(s). However, organisms will only reproduce, if they have some urge (conscious or unconscious) to do so. Therefore, apart from these processes, the variation of hormone concentrations is important for the mental status and behaviour of the individual. In fact, it is now well established that sex-steroids, including 17β-estradiol and its receptors, are present in brain areas beyond the hypothalamus (and the pituitary), not directly involved in reproduction, since many higher processes are required for this overall aim (see McEwen, 2002 a). Indeed, it is not difficult to imagine the importance of e.g. an intact memory, or increase in attention, when it comes to taking care of, and defending the offspring, which involves brain structures such as e.g. the hippocampal formation (HiFo) and the cerebral cortex. Since steroid hormones – including 17β-estradiol – exert powerful effects on manifold biological processes including chemical neurotransmission, there is a comprehensive interplay of all involved neurotransmitters and neuromodulators, which is intricate and may differ between females and males. However, the intricate effects of sex hormones on the brain *milieu internal* is far from completely understood.

Role of neuropeptides

Neuropeptides are amongst the phylogenetically oldest messengers known (see Johnsen 1998; Larhammar & Salaneck, 2004). The concept that neuropeptides produced in the brain and gut have direct effects on neurons was initially formulated by de Wied, Kastin and coworkers (see Kastin et al., 1983; de Wied 1984).

Neuropeptides affect both non-neural tissues and neurons, and they may be particularly important for integrative processes in cells and organs (see Strand, 1999). They are known to co-exist with, and modulate the effects of, classical neurotransmitters, inclu-

ding monoamines and amino acids (see Hökfelt et al., 1980; see Merighi 2002). Several neuropeptides including galanin are also involved in the growth and maintenance of the nervous system. It is also established that steroids modulate neuropeptide production and release (see Merchenthaler et al., 1998; Moenter et al., 2003). Steroids are therefore likely modulators of neuronal functions in general and of neurotransmission in particular.

The three decade-long surge of discoveries of new mammalian neuropeptides starting in the late ninteen sixtees (see e.g. Schally 1970; Mutt 1976, 1978, 1980; Gullemin 1978; Leeman 1980) was followed by ongoing detailed studies of their complex biological roles in central/peripheral systems, including higher brain functions, homeostasis and in pathophysiological processes.

The basic hypothesis directing the present work has been that neuropeptides in the brain can be influenced by sex steroids, in fact even by the distinct changes in their concentrations during the ovarian cycle in the brain areas beyond those related to reproduction. This concept received support from work in our laboratory showing effects of gender and age and of 17β-estradiol on concentrations of several neuropeptides in the HiFo of ovariectomized rats (Rugarn et al., 1999 a, b). Neuropeptides may then exert direct transmitter- like effects, influencing functions such as memory, cognition and mood.

The hippocampal formation (HiFo)

Ever since the pioneering work of Brenda Milner and associates, the HiFo has been ascribed an important role in learning and memory (Scoville & Milner, 1957). However, the HiFo has also been referred to as a structure sensing changes in the body and connecting to the neuroendocrine system (see Lathe, 2001), that is being of importance, e.g. for emotion and stress (McEwen 2002 b).

The HiFo is a bilaminar grey-matter structure that in humans forms the floor of the inferior temporal horn of the lateral ventricle and extends from the anterior margin of the ventricular horn to the splenium of the corpus callosum (see Witter & Amaral, 2004). In the rat the HiFo is C-shaped with a septal and temporal pole and consists of the dentate gyrus (DG) and the hippocampus proper – Cornu Ammonis (CA). The latter is divided into the regions CA1 – CA3. The circuitry of the HiFo encompasses the following major pathways: 1) the *perforant pathway* – the main input – which mainly projects from the entorhinal cortex to the granule cells of the dentate gyrus; 2) the *mossy fiber pathway*, which projects from granule cells to the pyramidal cells in CA3 region; and 3) the *Schaffer collateral pathway*, which projects from CA3 pyramidal cells to pyramidal cells in the CA1 region. The CA1 pyramidal neurons in turn represent the output and project to the pyramidal neurons of subiculum, a relay which conveys information from the HiFo to the enthorinal cortex. In addition there are several populations of interneurons (see Freund & Buzaki, 1996). The main neuron populations in the HiFo are glutamatergic. The remaining 10% are primarily gamma-aminobutyric acid (GABA)-producing interneurons. HiFo also receives input from subcortical structures such as: 1) *cholinergic and GABAergic* neurons in the medial septum and the diagonal band that ramify extensively throughout the HiFo to release acetylcholine (ACh) and GABA, respectively acting on a diverse range of muscarinic and nicotinic ACh and GABA receptors; 2) a *noradrenergic* pathway from the locus coeruleus (LC) acting at adrenoreceptors; 3)

serotonergic input from the dorsal and medial raphe exerting their influence via various types of of serotonin receptors.

Hippocampal functions are manifold and involve a wide variety of messenger molecules and their receptors. From the view point of the present thesis the steroid receptors are particularly intriguing. Thus there is a high density of glucocorticoid receptors in the HiFo (McEwen et al., 1968). The glucocorticoids (cortisol in humans and corticosterone in rodents) are produced and secreted by the adrenal glands and play an important role in mobilizing the body for fight or flight responses. There is evidence that the HiFo plays a major role in regulating the secretion of glucocorticoids via negative feedback to the hypothalamus and the pituitary (Sapolsky et al., 1986). Damage to the HiFo can result in over-activation of the hypothalamic-pituitary-adrenal (HPA) axis, which, in turn, has been associated with cognitive impairments and hippocampal damage (McEwen, 2002 b). Rapid, non-genomic effects by glucocorticoids have also shown to alter neuronal activity (see Joels et al., 1997). There is now evidence that receptors for sex steroids are present in the HiFo. Thus, both estrogen receptor α (ERα) (see Jensen et al., 1996) and β (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996) have been shown to be expressed in subpopulations of hippocampal neurons (as well as in neurons projecting to the HiFo) both in rodents and in humans (Shughrue et al., 1997 a, b; Wieland et al., 1997; Österlund et al., 2001; Mitra et al., 2003; Merchenthaler et al. 2004).

Galanin

Galanin was discovered by the late Victor Mutt, Kazuhiko Tatemoto and co-workers based on presence of cleavable COOH-amide – a characteristic feature of many currently known biologically active peptides (Tatemoto & Mutt, 1978, Tatemoto et al., 1983). Rat and mouse galanin consists of the 29 amino acids Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH_2. Human galanin is not C-terminally amidated and includes the additional amino acid serine. Galanin is a peptide cleavage product of the preprogalanin produced from the preprogalanin gene (Vrontakis et al., 1987; Kaplan, et al., 1988). The promoter contains binding sequences for factors shown to be regulated by estrogen (Vrontakis et al., 1987).

Since its discovery, numerous investigations have shown tissue specific distribution of galanin in several neuronal systems in the brain, spinal cord, and in peripheral tissues of rats and mice (Rökaeus et al., 1984; Ch'ng et al., 1985; Melander et al., 1985, 1986 a; Skofitsch & Jacobowitz, 1985, 1986; Gundlach et al., 1990, 2001; Jacobowitz & Skofitsch, 1991; Ryan & Gundlach, 1996; see Jacobowitz et al., 2004). Galanin was furthermore shown to co-exist with monoamines, amino acid transmitters and with other neuropeptides in the rat central nervous system (CNS) (Melander et al., 1986 b) and is thought to participate in a wide range of physiological functions, mainly as studied in rat. Galanin has been shown to co-exist with noradrenaline (NA) in the LC (Holets, et al., 1988), and the majority of the NA fibers in the hippocampal formation contain galanin (Melander et al., 1986 c; Xu et al., 1998). However, biochemical studies combined with neuronal lesions suggest presence also in other systems such as the cholinergic and serotonergic pathways (Gabriel et al., 1995).

Galanin receptors

Molecular cloning studies have identified three different G-protein-coupled galanin receptors (GalR) (Habert-Ortoli et al., 1994; Burgevin et al., 1995; see Jacoby et al., 1997; Branchek et al., 1998, 2000; Iismaa & Shine, 1999) activating several intracellular signalling cascades. GalR1 is linked to a G_i protein subtype, and activation of this receptor inhibits the forskolin-mediated cAMP production (Wang & Gustafsson, 1998). GalR2 (Fathi et al., 1997) activation has been associated with elevation of inositol triphosphate (IP3) as well as a reduction of the forskolin-elevated cyclic adeno monophosphate (c AMP) concentrations (Kask et al., 1997; Wang & Gustafsson, 1998), suggesting that GalR2 activation in some cases can mimic GalR1-mediated effects. In situ hybridization data indicate that GalR2 is the dominant galanin receptor present in the dorsal HiFo (O´Donnell et al. 1999; Burazin et al., 2000), although there are some studies reporting expression of GalR3 mRNA (Kolakowski et al 1998; Waters & Krause 2000).

N-terminal galanin and the possible existence of other members of the a galanin family of neuropeptides

Initially, galanin(1-29) did not appear to share structural features with any other neuropeptide (Vrontakis et al., 1987), and was therefore assumed not to belong to any known peptide family. However, in 1999 the novel galanin-like, 60 amino acid long peptide GALP was cloned, and the amino acids in position 9-21 were shown to be identical with positions 1-13 in galanin(1-29) (Ohtaki et al., 1999). It has been suggested that other

members of the galanin family may exist. The four following observations are arguments for such homologues: 1) Galanin-like immunoreactivity (galanin-LI) was early on shown to be quite heterogeneous in extracts of the rat gastrointestinal tract (Rökaeus et al., 1984; Norberg et al., 2004). Rökeus et al., showed that tissue-extracts cross-reacted with antiserum raised against porcine galanin, also shown to be distinctly different from galanin(1-29) in chromatographic characterizations. This was also supported by using an antiserum raised against rat galanin (Theodorsson & Rugarn 2000); 2) the low affinity in the binding of galanin(1-29) to human GalR3 suggests that there are other endogenous ligands, structurally related to galanin; 3) more than ten years ago Hedlund and colaborators (1992) demonstrated, in quantitative receptor autoradiographic studies, a widespread distribution of ^{125}I-galanin(1-15) binding sites in the rat brain, e.g. in the dorsal HiFo; 4) the fact that three different galanin receptors have been cloned suggests that there could be a family of galanin-related homologues.

In electrophysiological studies, Xu and co-workers (1999) have shown, in a subpopulation of cells in the HiFo, a response selectively to galanin(1-15) but not to galanin(1-16) or galanin(1-29). In contrast, neurons in the LC responded to galanin(1-29) and galanin(1-16) but not to galanin(1-15). Since areas like the dorsal HiFo had been shown to have only very few binding sites for the parent peptide galanin(1-29) (Melander et al., 1988), these studies together indicated presence of a new type of galanin receptor selective for N-terminal galanin fragments.

The N-terminal portion of galanin(1-29) is highly conserved up to position 14 in all species investigated (see Rökaeus et al., 1987), and binds preferentially to the receptor (Lagny-Pourmir et al., 1989). This is in contrast to most other neuropeptides that bind to their receptor with their C-terminal end. Substitution of the individual amino acids in the N-terminal part of galanin(1-29) with Alanine have shown that Trp2, Asn5 and Tyr9 are important for receptor binding (Land, et al., 1991). Studies of galanin metabolism have shown that the C-terminus of galanin seems to protect the N-terminal portion from proteolytic attacks (Land et al., 1991; Bedecs et al., 1995).

However, there is so far no evidence that biologically active partial sequences of galanin are generated in vivo from endogenous galanin(1-29), and no naturally occurring galanin-related peptides with similar structure as the N-terminal part of galanin have been found.

Examples of the biological roles of galanin in the rodent nervous systems

Galanin plays numerous roles in the brain and in the peripheral nervous system. For a recent overview of the galanin field, see Hökfelt (2005). Although a prominent general function is inhibition of neuronal excitability, other effects have also been described, in turn modulated by the concentrations of other neurotransmitters in the environment, the receptor subtypes and transduction mechanisms. Below is a brief overview of its functions related to the HiFo and of interest for the present thesis.

Aqusition and retention

Galanin attenuates neuronal long term potentiation (LTP) (Sakurai et al., 1996; Mazarati et al., 2000; Coumis & Davies, 2002) and has been shown to impair spatial learning in the rat, an effect suggested to at least partly be due to reduction of ACh in the extracel-

lular fluid (ECF) (Fisone et al., 1987, Ögren et al., 1996). However, galanin infused into the medial septum increases hippocampal ACh in the ECF and improves spatial learning, suggesting that galanin in medial septum excites hippocampal cholinergic neurons (Elvander et al., 2004). Galanin and GalRs are overexpressed in brain areas associated with cognition in Alzheimer´s disease (AD), suggesting a role for the galanin system. However, the functional consequences of this overexpression are still being investigated (see Schött et al., 1998; Crawley, 1996; Counts et al., 2003; Mufson et al., 2005; Rustay et al., 2005).

Mood and stress

Galanin has been ascribed roles in stress-related responses. The firing rate of noradrenergic neurons in the LC is attenuated by galanin, leading to subsequent hyperpolarization (Xu et al., 2005). This was also shown in 5-hydroxytryptamine (5HT) (serotonin) neurons in the dorsal raphe nucleus (DRN) (Xu et al., 1998). Furthermore, microdialysis studies of the ECF in the rat HiFo show that galanin causes a reduction of extracellular 5HT when galanin is injected intraventricularly and into the DRN in both rats and mice (Kehr et al., 2001; 2002).

Galanin has also been implicated in epileptic disorders (see Mazarati, 2004), feeding behavior (see Leibowitz, 2005), injury and neuronal repair (see Holmes et al., 2005, Shen et al., 2005) and in pain (see Wiesenfeld-Hallin et al., 2005). However, this is far from a complete list of functions related to galanin.

Neuropeptide Y

NPY, another neuropeptide discovered in the laboratory of Viktor Mutt, is a 36-amino acid peptide, initially isolated from tissue extracts of the porcine brain (Tatemoto 1982; Tatemoto et al., 1982). NPY is, together with peptide tyrosine, tyrosine (PYY) and pancreatic polypeptide, a member of the pancreatic polypeptide family, and one of the most conserved peptides amongst species (see Larhammar, 1996), and highly abundant in the brain (Allen et al., 1983; Chronwall et al., 1985; de Quidt & Emson 1986 a, b; Gehlert et al. 1987). NPY has been shown to co-exist with catecholamines in some cell groups in the rat CNS (Everitt et al., 1984). In the HiFo, NPY coexists with GABA in interneurons both in the ventral and dorsal parts (see Freund & Buzsaki, 1996).

Neuropeptide Y receptors

Five NPY receptors have until now been cloned, all members of the 7-transmembrane G-protein-coupled receptor family (see Larhammar & Salaneck, 2004). All NPY receptors seem to couple in the same manner to G-proteins, primarily activating G_i which causes inhibition of adenylyl cyclase, but additional signal-transduction systems may be involved (see Gehlert 1998; Larhammar et al., 2001). The receptor Y_1, Y_2 and Y_5, are mainly expressed in the CNS, while Y_4 is expressed almost exclusively in the gastrointestinal tract in mammals (see Gehlert, 1998; Larhammar & Salaneck, 2004).

NPY receptor 1 (Y_1), first cloned in rat by Eva et al., (1990), is to date the best characterized receptor, exhibiting 93% homology with the human receptor (see Larhammar, 1996). Y_1 predominates in the cerebral cortex, thalamus and amygdala, although species differences were early on reported (Eva et al., 1990). Y_1 requires the full molecule of NPY and PYY for its activation while having lower affinity for C-terminal fragments such as NPY 3-36 and PYY 3-36 (Dumont et al., 1994). The NPY receptor Y_2 (Y_2), cloned by Rose et al., (1995) is distinguished from Y_1 by its preference for C-terminal fragments of NPY, and binds PYY with similar affinity (Fuhlendorff et al.,1990). Human and rat Y_2 exhibit a high degree of homology (98%). In the brain, Y_2 is found in the HiFo, substantia nigra, thalamus, hypothalamus and in the brain stem (Gehlert 1992; Dumont, 1994). The longer C-terminal sequences NPY 3-36 and PYY 3-36 were proposed to be selective Y_2 agonists, but they also have equal affinity for Y_5. The less studied Y_3 receptor has high affinity for NPY, but in contrast to Y_2, lower affinity for PYY (Glaum et al., 1997). The Y_4 receptor was cloned by several groups (Bard et al., 1995; Gregor et al., 1996; Lundell et al., 1995) and exhibits only 75% homology compared to human (Lundell et al., 1996) and has a higher affinity for PYY than for NPY (Eriksson et al., 1998; Berglund et al., 2001). The Y_5 receptor was isolated from rat hypothalamus (Gerald et al., 1996; Hu et al., 1996) and is very conserved (88-90%) across mammallian species. Additional subtypes of NPY receptor binding sites in the rat brain have recently been suggested by Dumont and co-workers (2005).

Examples of the biological roles of NPY in the rodent nervous systems

NPY plays a role in numerous important physiological processes, both in the CNS and in the peripheral nervous system.

Mood and stress

One of the first reported CNS actions of NPY was a long lasting synchronization of the

EEG pattern (Fuxe et al., 1983) which mimics effects of established sedative/anxiolytic compounds such as bensodiazepines or barbiturates (Ehlers et al., 1997). It is now well established that centrally administered NPY induces potent anxiolytic effects in both rats (Kask et al., 2002; see Heilig, 2004) and mice (Karlsson et al., 2005).

Electroconvulsive stimuli and administration of the clinically established stabilizer of affective functions lithium, induce up-regulation of hippocampal NPY levels (Stenfors et al. 1989) and synthesis (Husum et al., 2000). Evidence for an involvement of NPY in depression comes amongst others from the finding of differential NPY expression in genetic animal model of depression (Caberlotto & Hurd, 1999). The effects are brain region specific and the HiFo appears to be a candidate structure for a possible functional involvement in depression.

Memory and cognition and other functions

In the HiFo, NPY is mainly localized to GABA interneurons (see Freund & Buzsaki, 1996)), but is also present in cholinergic neurons (Milner et al., 1997). Septal cholinergic de-afferentation was shown to result in loss of a distinct subpopulation of hippocampal NPY-containing neurons. Furthermore, studies have shown a significant decrease of NPY-LI in cortical, amygdaloid and hippocampal areas in AD (Beal et al., 1986). However, the mechanisms underlying the decrease in NPY in cognitive disorders remain to be further established (see Heilig, 2004).

The role of NPY in feeding behaviour (Kalra et al., 1988; see Hökfelt et al., 1999), neuroproliferation (Howell et al., 2005) and epilepsy (Baraban, 2004; Woldbye & Kokaia, 2004) – to mention only a few – is well established.

Co-existence and co-release of neurotransmitters in the brain

The mechanisms of synthesis, storage, release, reuptake etc. of neuropeptides are markedly different from that of "classical" transmitters such as monoamines and amino-acids (see Hökfelt et al. 1980; Zupanc 1996, Merighi et al., 2002).

Neuropeptide synthesis

Neuropeptides are synthesized on ribosomes in the nerve cell soma as large precursor molecules (prepro- and propeptides), cleaved into appropriate size and posttranslationally modified by specific enzymes (see Strand, 1999). They are stored in large dense core vesicles (LDCVs) and centrifugally transported to the nerve terminals. In contrast classical transmitters can be synthesised both in the soma and within the nerve endings and are stored in small synaptic vesicles (SSV) and, at least in some cases, also in LDCVs. Some SSVs are located in close apposition to the active zone, whereas the LDCVs mainly are located at some distance from the active zone, and are mobilized and recruited for exocytosis tethered to cytosolic elements such as actin (see Shakiryanova et al., 2005).

Neuropeptide release

The kinetics of the LDCVs appear to vary considerably depending on the cell type, release site and the messengers involved (Seward et al., 1995). In the peripheral nervous system the release of neuropeptides has been found to be dependent on the stimulus frequency (Lundberg 1981, 1996; Lundberg et al. 1982, 1983, 1986). Interestingly, whereas action potentials originating at the neuronal soma trigger neuropeptide release from terminals, Ca^{+2} released from intracellular stores can result in independent release from dendrites (Ludwig et al., 2002). Thus, the release from SSVs versus LDCVs is suggested to be differently regulated (see e.g. Verhage et al., 1991).

Other mechanisms in addition to changes in the release of the neuropeptides from LDCVs into the extracellular fluid, may play a role in modulating the effects of peptidergic neurons. This includes the enzymatic degradation of peptides after their release and receptor binding and possible re-uptake of neuropeptides into the nerves. According to a widely accepted paradigm there is no re-uptake of neuropeptides after fusion of the LDCV with the cell membrane and transmitter release. However, there is a study suggesting re-uptake of calcitonin gene-related peptide (CGRP) into nerve terminals (Sams-Nielsen et al., 2001). In addition there are rapid effects on neuropeptide synthesis near to the site of release. Indeed, there is evidence for presence of neuropeptide mRNA pools in nerve terminals of the posterior pituitary originating in the hypothalamic magnocellular supraoptic and paraventricular nuclei (Trembleau et al., 1996, Mohr et al., 2002), possibly also for galanin mRNA (Landry & Hökfelt, 1998). In this case galanin may be synthetized close to the site of release, allowing synthesis close to the release sites, which might be an additional mechanism for modulating peptidergic transmission.

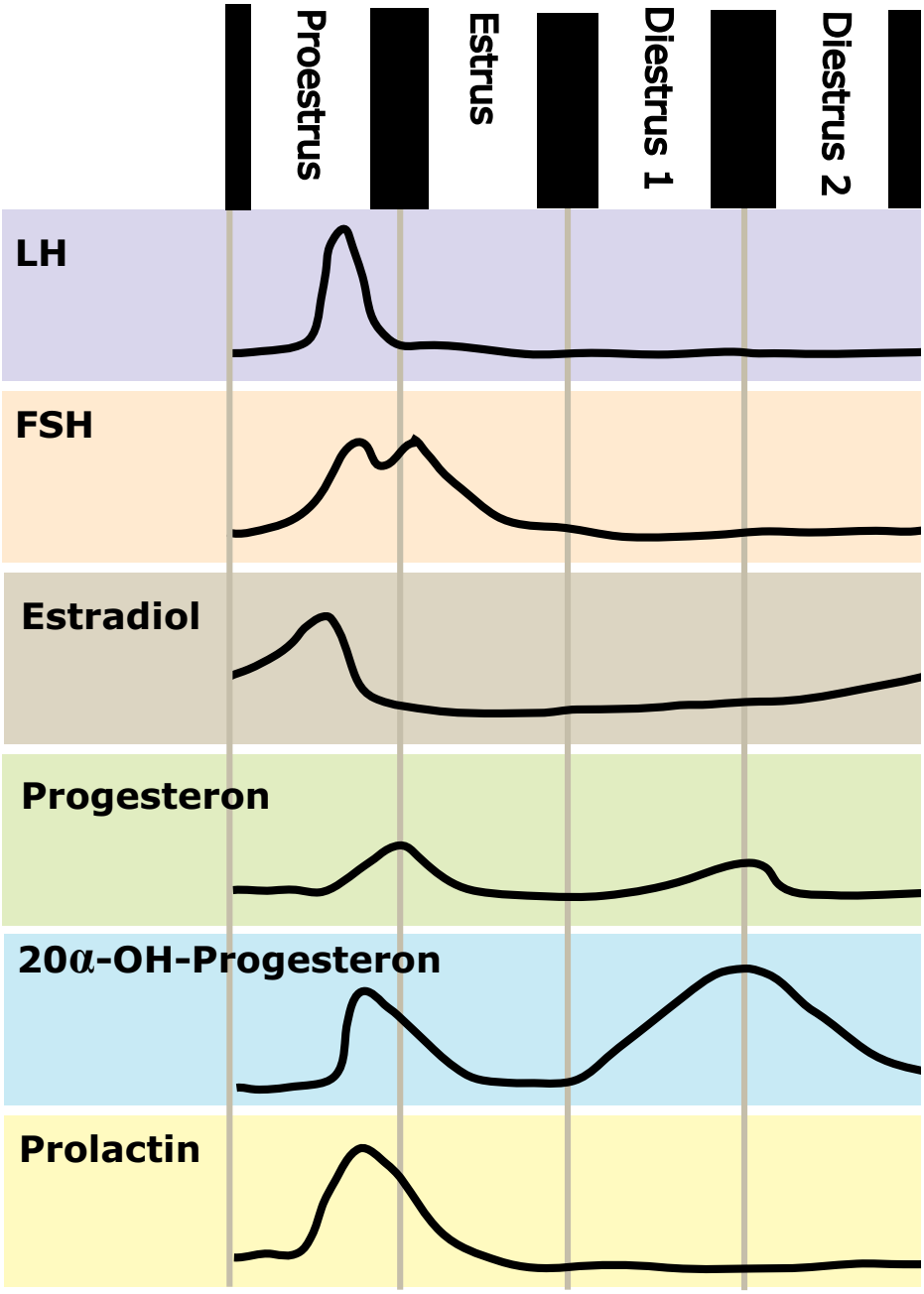

Figure 1

A graphic representation of a 4 day estrous-cycle in rats representing plasma concentrations of estradiol, progesteron, prolactin as well as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Adapted and modified from Knobil et al., 1994.

Estrogens

Synthesis

Androgens and estrogen are derived from cholesterol starting with the rate limiting conversion to the precursor pregnenolone by cytochrome p-450 and further a conversion to progesterone (see Knobil et al., 1994). In a further step, androgens are synthesized by hydroxylation of progesterone to androstenedione (an androgen), and the reduction of a methyl group and formation of an aromatic A ring then forms estrogen. The cytochrome p-450 aromatase enzyme complex (Naftolin & MacLusky 1982), involving multiple, specific cytochrome P-450 containing enzymes, is differently distributed in organelles, cells and organs (Jefcoate et al., 2000). In females, estrogens (Falck 1959) and progestins (Allen & Wintersteiner, 1934) are mainly, but not exclusively, produced by the ovaries (see Knobil et al., 1994). In the mammalian brain, there are two separate aromatase systems; a gonad-sensitive hypothalamic system and a gonad-insensitive limbic system (see Naftolin 1994). Amongst the estrogens, 17β-estradiol has the dominating endogenous biological activity. The production of all ovarian hormones in both rodents and human is controlled by the pituitary hormones luteninzing hormone (LH) and follicle-stimulating hormone (FSH) which in turn are regulated by gonadotrophin-releasing hormone (GnRH) (see Bousfield et al., 1994) (Fig. 1) synthetized in the hypothalamus.

Estrogen receptors

Mueller and co-workers showed early (1957) that estrogen enhances the biosynthesis of phospholipids, nucleic acids and proteins in the rat uterus (see Knobil et al., 1994). Further on it was found that both estrogen and progesterone regulate mRNA and protein synthesis, leading to the conclusion that estrogens have direct effect at the level of RNA production. According to todays knowledge, estrogen diffuses easily through the cell membrane and binds to specific receptors within the cytoplasma or at the nucleus (see Jensen et al., 1996; Koehler et al., 2005). Without ligand the receptor associates with a large complex of chaperones (heat-chock proteins) and then dissociates by phosphorylation upon ligand-binding. The estrogen/receptor complex then form dimers resulting in a conformational change - turning the complex to an active structure with abilities to bind estrogen responsive elements (EREs), which are regulatory DNA-sequences (see Matthews & Gustafsson; 2003).

Estrogens actions in the brain

The first detailed insights into how estrogens influence the organism were made possible by the availability of hormones labeled with radioactive isotopes, enabling the monitoring of the transport from the site of synthesis in the endocrine glands, through the blood circulation to the target tissues (see Jensen, 1996). Subsequent binding-studies demonstrated specific high-affinity steroid receptor complex that could induce expression of hormone induced genes (see Jensen, 2005). The estrogen receptor first discovered is now called ERα. When Jan-Åke Gustafsson and co-workers (Kuiper et al., 1996; Mosselman et al., 1996) more recently discovered the second estrogen receptor (ERβ) it was a reason to re-evaluate many of the earlier conclusions about estrogen effects, e.g. how to use estrogens more appropriately for therapeutic purposes (see Barkhem et al., 2004). It was found that both ERs share common structural and functional domains, bind to estrogen with high affinity, and bind EREs in a strictly regulated manner, but they differ

in many ways in respect to their tissue distribution and transcriptional activities.

Thus, using immunohistochemistry and *insitu* hybridization techniques, the mRNA and immunoreactivity of ERβ were shown to be more widespread than those of ERα. Thus ERβ is found in the cerebral cortex, HiFo and the cerebellum, as well as in the cardiovascular and immune systems and other tissues in both rodents and in humans, tissues traditionally not considered to be directly involved in reproduction (Shughrue et al., 1997; Weiland et al., 1997; Österlund et al., 1998; Österlund & Hurd, 2001; Mitra et al., 2003; Merchenthaler et al., 2004). It is now very well recognized that 17β-estradiol acts on a variety of physiological parameters both in the brain (McEwen, 1987; 2002 a, b) and in the pheripheral nervous system (Amandusson et al., 1999). It is, however, as yet not clear what this difference in the distribution of ERα and β means for the functions of HiFo and neurotransmission processes. Further studies in this area will be challenging e.g. since distinct differences whithin the same species (e.g. in LC) are reported (Mitra et al., 2003; Vanderhorst et al., 2005) as well as between species such as e.g. in the serotonergic neurons in the DRN (Sheng et al., 2004).

Estrogens also activate a variety of alternative signaling pathways, not directly involving the cell nucleus, some of which are independent of the classical nuclear estrogen receptors ERα and ERβ (Kelly et al., 1976, 1977; Pietras & Szego, 1977; see Kelly & Levin, 2001; Toran-Allerand, 2005). In the present thesis, we use a simplified distinction between the classical nuclear ERs (ERα and ERβ), acting by regulating the expression of specific genes through EREs (**genomic**), and effects mediated by other signaling pathways acting rapidly on transcriptional factors other than the ERE (e.g. activator protein 1 (AP 1) and cAMP-responsive element (CRE)) (**indirect genomic**) and effects not depending on transcription, translation, and productions of proteins (**non-genomic**), e.g. direct interaction with ion-channels (Fig. 2). These non-genomic effects share the following characteristics: 1) the responses are too rapid to be generated by de novo protein synthesis (occurs within minutes); 2) they can be reproduced in the presence of inhibitors of RNA or protein synthesis; and 3) they can be reproduced in cells by estrogen coupled to a membrane-impermeable molecule (e.g. estradiol plus bovine serum albumin (BSA)).

Since the receptors and signaling elements for the rapid indirect genomic and non-genomic effects of estrogens are not yet fully characterized, the nomenclature currently used in the field is not sufficiently harmonized and precise. It is likely to change as new details in the receptors and signaling systems are revealed.

Rapid effects of steroids on the brain

Estrogen exposure, acting on the classical nuclear receptors ERα and β, changes the expression of genes and synthesis of the proteins they encode. The resulting fluctuations in the amounts/levels of proteins underlie the overall physiological response that takes place hours following estrogen exposure (McEwen et al., 1987).

However, steroids act also in a rapid manner. In fact, the first reported ‘rapid’ effect of steroids on the brain was the immediate anaesthetic effect of progesterone demonstrated in 1942 by Hans Selye (Selye, 1942 a, b). Later these rapid effects were also shown to be induced by 17β-estradiol (Kelly et al., 1976, 1977, Pietras & Szego, 1977). Rapid,

non-genomic effects – possibly mediated through G-protein coupled mechanisms – was subsequently shown in the rat HiFo by means of electrophysiology (Gu & Moss, 1996). The ER-independent mechanism of the effect was supported also in ER knock-out mice by the same group (Gu & Moss, 1999). These results suggest a role for estrogen in the modulation of excitatory synaptic transmission in the HiFo mediated by the G-protein, c-AMP cascade. Furthermore, rapid effects of 17β-estradiol in rats on the immediately early gene c-Fos was also demonstrated by Rudick and co-workers (Rudick & Woolley, 2003). They found that 17β-estradiol fails to increase c-Fos at 2 h in the ventral hippocampus, where many pyramidal cells express a nuclear ER, but increases c-Fos in CA1 pyramidal cells of the dorsal HiFo – a brain region which expresses very few nuclear ERs (Shughrue et al., 1997; Hart et al., 2001). However, Abraham et al., (2003) found rapid effects of 17β-estradiol on phosphorylated -cAMP responsive element binding protein(CREB) in the mouse HiFo, dependent on ERβ.

Of functional interest in the view of these findings are the rapid effects of 17β-estradiol on the HiFo as shown in the electrophysiologic studies by Terasawa et al. (1968) in the HiFo, demonstrating that the seizure threshold decreases in the afternoon of proestrous, when the estrogen plasma levels peak.

Evidence has been presented for the presence of a novel estrogen receptor – ER x – located in the cell membrane and interacting with other membrane proteins to initiate a number of alternative signaling cascades and subsequent rapid intracellular responses (Toran-Allerand et al., 2002). This receptor may be localized within small vesicular invaginations of the plasma membrane called caveolae (see Toran-Allerand, 2005), onto which this novel estrogen membrane receptor and other signaling molecules dock. Forcing a variety of molecules into close proximity, caveolae are suggested to expedite the activation by estrogen of the Ras–Raf–mitogen-activated protein kinase (MAPK) cascade. MAPKs are strongly activated by neurotrophins and neurotransmitters (see e.g. Grewal et al., 1999) and have been implicated in the cellular and molecular mechanisms of various forms of memory (Thiels et al., 2002), cell growth and differentiation (Wade et al., 2001).

Estrogens also influence the morphology of pyramidal cells in the hippocampal CA1 region by stimulating the growth of dendritic spines and the genesis of new synapses, changes that are paralleled by an increase in N-methyl-D-aspartate (NMDA) glutamate receptor function (Frankfurt et al., 1990, Woolley & McEwen, 1992, Woolley et al., 1997).

Taken together, present evidence indicates that the biological effects of 17β-estradiol are induced by receptors and signaling mechanisms in addition to the classical nuclear/genomic effects. In the present context the effects of estrogens on neurotransmitters in general, and on neuropeptides in particular, are especially intriguing (see Merchenthaler, 2005).

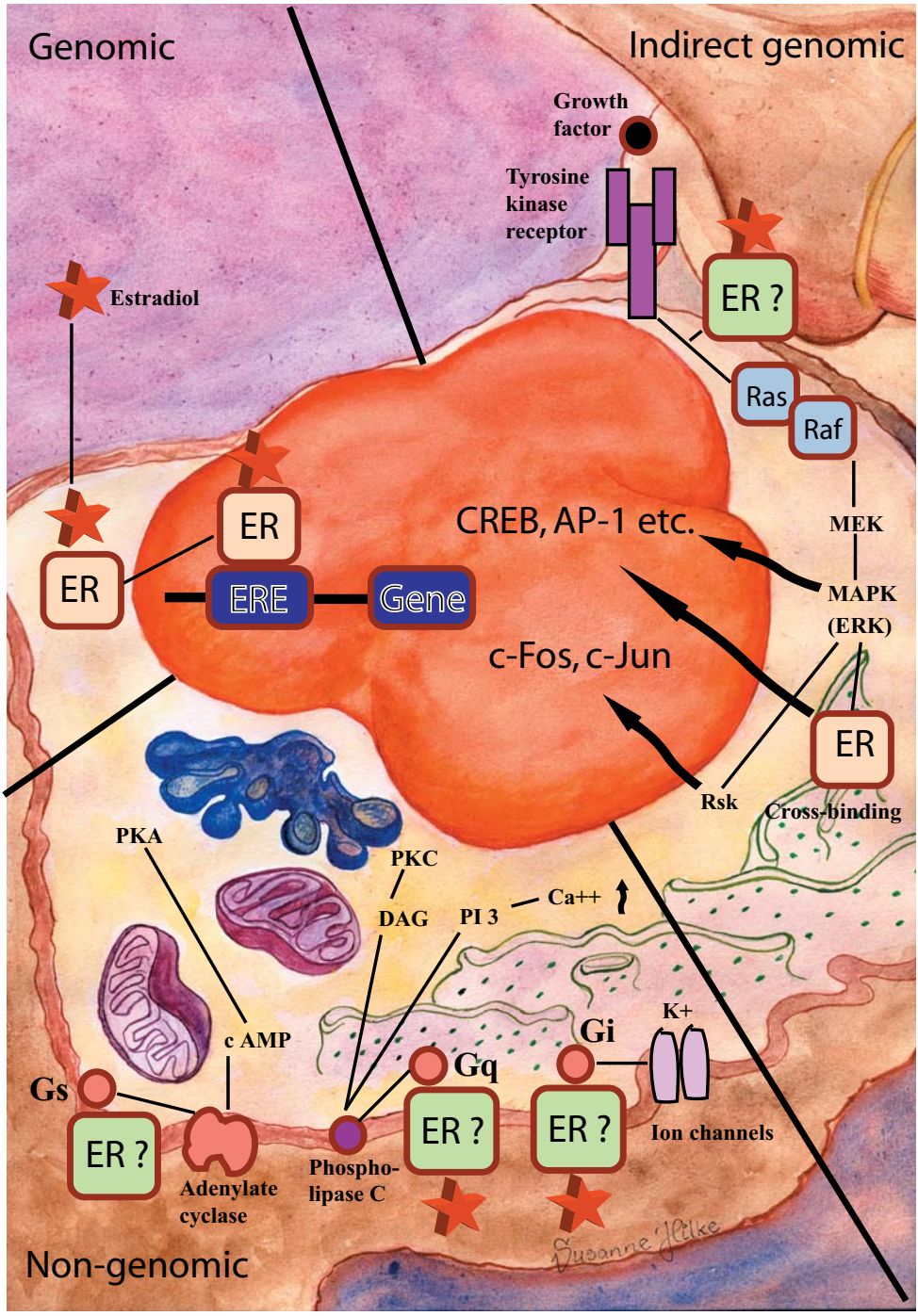

Figure 2

A graphic illustration of some aspects of the genomic, indirect genomic and non-genomic mechanisms of estradiol.

Functional aspects of hippocampal galanin, NPY and estrogens

The functional implications of estrogen in brain areas related to memory, cognition, aurosal and mood are plentiful (McEwen, 2002 a, b), and the effects of estrogen and galanin, and NPY earlier presented separately show considerable overlaps;

1) Estrogen, galanin and NPY have all been shown to modulate excitability in the rodent HiFo. Galanin and NPY inhibit glutamate release though presynaptic mechanisms (Colmers et al., 1988; Ben-Ari 1990), and both have been shown to attenuate or inhibit the generation of LTP (Whittaker et al., 1999; Coumis & Davies 2002; see Mazarati 2004). Estrogen, on the other hand, acts in the opposite functional direction in the HiFo, enhancing the excitability in distinct populations of cells (Woolley et al., 2000; Foy et al., 2001).

2) Galanin in the cholinergic forebrain neurons is co-localized with choline acetyltransferase (ChAT) (Melander et al., 1985, 1986 c), inhibits ACh release in the HiFo (Fisone et al., 1987), and impairs cognitive performance (see Crawley 1996; Ögren, 1996; Schött et al., 2000), suggesting a possible role of galanin in acquisition and retention and a possible significance in AD (see Crawley, 1996, Ögren et al., 1999; Counts et al., 2003), although acting in an opposite direction when injected in the medial septum (Elvander et al., 2004, see Elvander 2005). NPY is to some extent present in cholinergic neurons (Milner et al., 1997) and significantly reduced NPY-LI is found in cortical, amygdaloid and hippocampal areas in AD (Beal et al., 1986). On the other hand, estrogen increases the density of dendritic spines in the rat HiFo (Frankfurt et al., 1990) in the pro-estrous phase (Woolley & McEwen 1992), and is suggested to improve memory and cognition in animal models (Luine et al., 1998; Bimonte et al., 2000),

3) A growing body of evidence supports a role for galanin (Xu et al., 1998; Kuteeva et al., 2005) and NPY (Redrobe et al., 2002; see Heilig, 2004) in the mediation of stress and anxiety. Galanin influences 5HT receptors both at the levels of the cell bodies in DRN and in forebrain areas such as the HiFo (see Fuxe et al., 1998) and inhibits 5HT and NA release in the HiFo (Yoshitake et al., 2003; 2004). Low levels of plasma and cerebrospinal fluid NPY have been found in patients with depression and anxiety disorders (Ekman et al., 1996; Nilsson et al., 1996), while galanin binding is upregulated in the LC in response to both acute and chronic restraint stress in male rats (Sweerts et al., 2000) 17β-estradiol modulates catecholamine biosynthesis in the LC (Serova et al., 2005). Increased release of NA in the brain has been implicated in mood disorders including depression (see Delgado & Moreno 2000). The relation between 17β-estradiol and stress seems to be dose- and time-dependent (Young et al., 2001). Thus, physiological doses of 17β-estradiol inhibit the responsiveness to stress, whereas blocking the effects of estradiol in gonadally intact female rats leads to exaggerated stress responses.

4) Estrogen has been shown to stimulate neurogenesis in the DG of adult female rats (Tanapat et al., 1999; 2005). In fact, short-term but not long-term treatment with 17β-estradiol was shown to stimulate neurogenesis in the DG of adult female rats. Furthermore, female rats show a lower degree of dendritic atrophy in the HiFo than male rats, suggesting that estrogen acts as a trophic factor in the HiFo (Galea et al., 1997). Galanin exerts neurotrophic actions in the adult male rat brain during growth and in response to

injury (Burazin & Gundlach, 1998; see Wynick et al., 2001), and the galanin receptors (GalR1 and GalR2) are located in the stem cell rich areas of the DG in the adult brain (Shen et al., 2003). However, surprisingly, isolated neural stem cells from the subgranular zone of the DG show decreased cell proliferation/survival in response to galanin (see Shen et al., 2005). NPY has been shown to stimulate growth and proliferation in the olfactory bulb (Hansel et al., 2001) and induces an increase in total cell counts and cell proliferation in the HiFo (Howell et al., 2005).

However, the effects of estrogen and its interactions with neuropeptides, e.g. by modulation of other classical neurotransmitters (e.g. NA etc.) in HiFo, are only in the early stages of investigation.

Hypothesis

The main focus of the present thesis is on how galanin and NPY systems are affected by the female sexhormone 17β-estradiol, both in estrogen-treated ovx rats and mice and during a normal estrous cycle in rats .

Earlier findings from our laboratory, showed age- and gender dependent changes in the concentrations of several neuropeptides in the rat brain (Rugarn et al., 1999a), indicating involvement of neuropeptides in brain communication related to the normal variation of sex-hormones. This work showed that the levels of galanin and NPY are changed in the HiFo and cortex – areas not directly related to reproduction – also after *long-term* treatment for several weeks with 17β-estradiol to ovx rats (Rugarn et al. , 1999 b), indicating that galanin and NPY may be involved in hippocampal and cortical functions influenced by estrogen.

The main idea in this thesis work is that, in addition to the influences on galanin and NPY levels after *long-term* exposure to 17β-estradiol – most likely to be mediated through the classical ER pathway, this steroid can also affect neuropeptide levels in a *rapid* fashion, perhaps through a membrane-related mechanism. These rapid changes of neuromodulators could subsequently affect classical neurotransmitters that more directly control the synaptic plasticity in brain areas related to cognitive functions such as the HiFo and cortex.

This hypothesis is also extended by the idea that there are other members in the galanin family - yet not identified - with similarities in binding properties to the N-terminal part of the peptide and with the ability to modulate neurotransmission in the brain. To test this hypothesis we raised antibodies against the C-terminal part of galanin(1-16) in rabbits with the aim to identify further members of the galanin family.

Aims

To

- investigate the effects of dose and duration of 17β-estradiol treatment on galanin concentrations in the female rat and mouse HiFo.
- study rapid (hours) effects of 17β-estradiol on hippocampal galanin- and NPY-LIs and on gene expression in rats and mice.
- raise antisera against the C-terminal portion of galanin(1-16) in rabbits in order to identify putative endogenous molecules with structural similarities to the N-terminal part of galanin
- explore the possible occurrence of additional galanin family members in various rat tissues, particularly the brain, by means of these antisera and a radioimmunoassay, as well as gel-filtration and high performance liquid chromatography.

Material and methods

Animals and surgery

Housing

Female Sprague Dawley rats (body weight 250-350g) and female Balb/C mice (body weight 22-25g; five weeks old) (all from B&K, Universal, Stockholm, Sweden) were kept at constant temperature (21±1°C) with free access to rat and mouse chow and water (Lactamin, Kimsta, Sweden) under a controlled 12 h dark/12 h light cycle (light on at 8.00 am). For studies of the effects of hormonal changes during the estrous cycle, the animals were housed in a separate room with reversed 12 h light/12h dark cycle (light on at 8.00 p.m.). The studies and their experimental protocol were designed according to the guidelines of, and approved by, the local ethics committee on animal research in Linköping.

Ovariectomy

The anaesthesia used in the present experimental surgery on rats and mice was in most cases 0.5-1.5% isofluorane, Forene (Abbott Scandinavia, Kista, Sweden) in an oxygen/nitrous oxide mixture (30%/70%). However, anaesthesia was in some experiments achieved with intraperitoneal injections of xylazin 12 mg/kg and ketamine 80 mg/kg (**paper II**). The ovaries were retracted from the abdominal cavity using the dorsal route. The junction between the fallopian tube and the uterine horn was sutured, the ovaries were carefully removed, and the uterine horns were reinplanted in the abdominal cavity. The animals were left for a two-week washout period, in order to eliminate circulating estradiol levels.

Administration of 17β-estradiol

To investigate rapid (one hour) effects of 17β-estradiol (Sigma, Aldrich, Sweden), a single injection was administered subcutanously under the skin of the back of the lower neck of the rats and mice. For studies of the effects of prolonged treatment with 17β-estradiol, slow-release pellets were used (Innovation Research of America, Sarasota, USA, http://www.innovrsrch.com/). The concentrations of 17β-estradiol achieved in plasma in response to treatment were similar to those obtained in the pro-estrous phase and during pregnancy, but in some instances the concentrations were pharmacological.

Administration of the selective estrogen receptor modifier Tamoxifen®

Tamoxifen citrate (Tocris, Bristol, UK) was dissolved in 99% ethanol and dimethyl sulfoxide and subsequently diluted in physiological saline. It was administered (1.5 mg/kg) 30 min before the 17β-estradiol in order to investigate, whether or not it blocked certain effects of estrogen.

Analysis of estrous cycles by vaginal smear

By monitoring the vaginal smear we made sure that all rats had at least two regular 4-day-cycles before starting the experiments. Cytology of vaginal smears was monitored daily according to the following schedule; 7.00-8.00 a.m. (light), *proestrous phase*, characterized by nucleated cells and lack of leukocytes; 1.00-2.00 p.m. (dark) *estrous phase*, swollen cornified cells lacking nuclei; 3.00-4.00 p.m. (dark), *diestrous phase*,

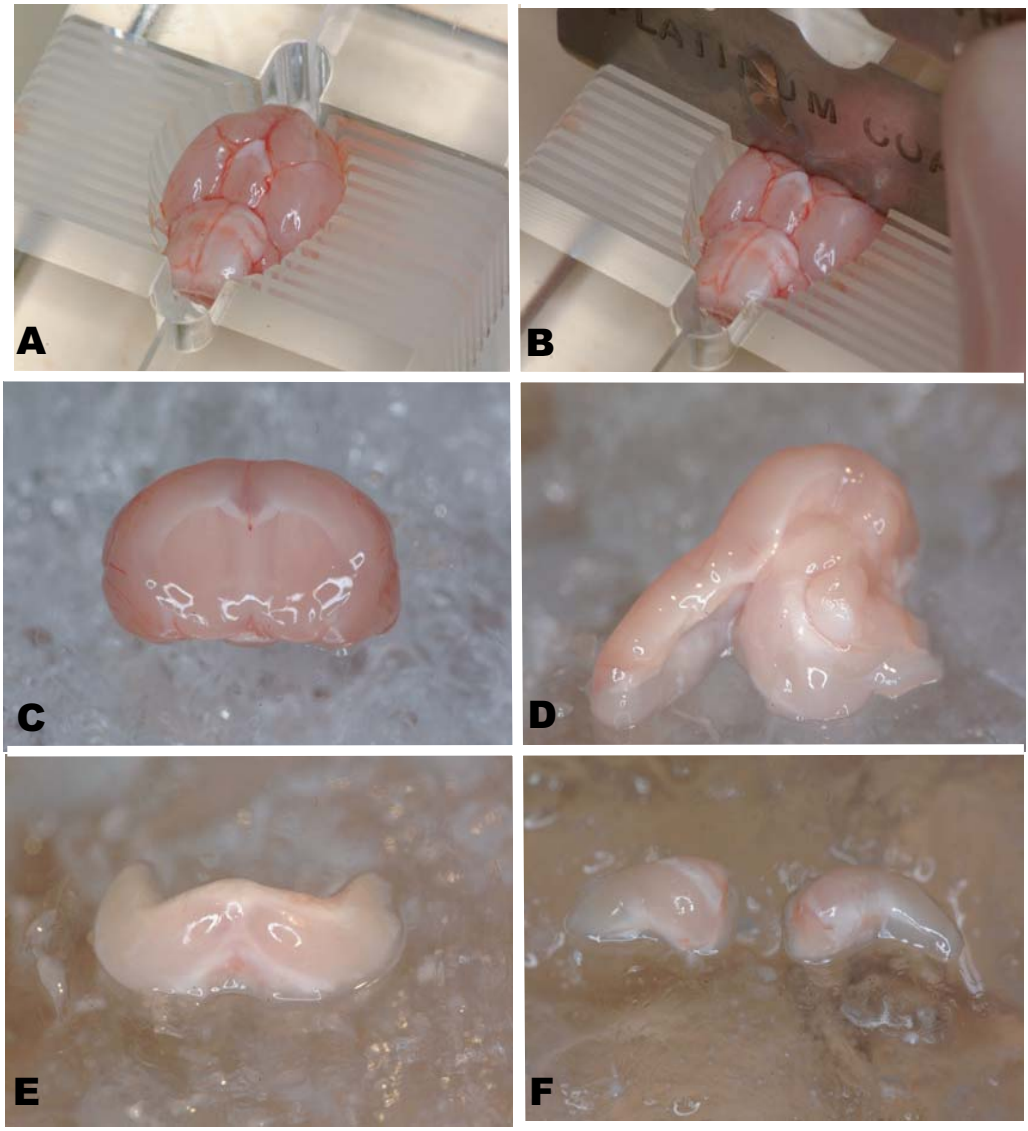

Figure 3

Photographs showing the detailed procedure of dissecting the hippocampal formation and other brain regions in mice

numerous leucocytes and nucleated cells.

Brain dissection

The dissection procedure in both rats and mice in all papers was modified from that described by Glowinski and Iversen (1966) (Fig. 3). First, the rhombencephalon was separated from the rest of the brain, the cerebellum was removed and the remaining piece (pons plus medulla oblongata) – termed medulla oblongata – was used for analysis. Next, using the optic chiasm as a landmark, a transverse section was made, creating the anterior limit for the 'hypothalamus', whereby the anterior commisure was the

dorsal horizontal border. In the rat the lateral border was approximately 2 mm from the midline. From this (approximately 5 mm thick slice), the hypothalamus was dissected. In the next slice (approximately 2 mm thick in the rat), the striatum (caudate nucleus – putamen) was removed (Fig 3 C). This was followed by blunt separation of the whole section of parietal, occipital and temporal cortex (Fig. 3 D). The left and right HiFo were then separated from the midbrain as shown in Fig 3 E. In papers **II** and **IV** the HiFo was divided in a dorsal and ventral portion. The frontal cortex piece was then removed from the remaining frontal part of the brain.

Microdialysis

An '*in vitro*' recovery experiment was performed prior to the proper study of the '*in vivo*' release of galanin in the rat HiFo. Dialysis probes (35kD, MAB 2.14, Microbiotech AB, Stockholm, Sweden) were immersed in Eppendorf vials containing standard concentrations of synthetic galanin(1-29) (250 and 1000 pmol/L) and perfused with Krebs-Ringer solution (138 mmol/L NaCl, 5mmol/L KCL, 1 mmol/L $CaCl_2$ 11 mmol/L $NaHCO_2$, 1 mmol/L NaH_2PO_4) containing 0.2% bovine serum albumin (Sigma) and 0.03% of the peptidase inhibitor Bacitracin® (Sigma). The perfusate was collected for one hour in 37°C at the flow rate 1 µL/min and 4 µL/min, respectively (Microbiotech AB). Microdialysates and samples of the outer medium – representing the total galanin concentrations – were then measured by means of RIA and the relative recovery calculated.

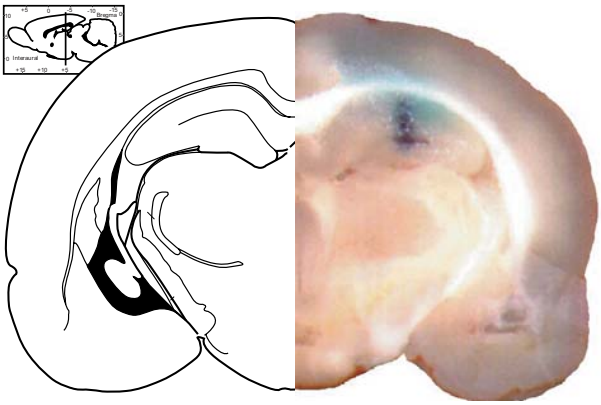

Figure 4

The position and verification of the position of the microdialysis probe in the dorsal HiFo during sampling.

For analysis of the *in vivo* release of galanin, the animals were anaesthetised, intubated with a tracheal cannula and placed in a stereotaxic frame for microdialysis experiments. The body temperature, heart rate and blood pressure were continuously monitored and blood gases were measured at the beginning of the experiment to regulate the tidal volume and frequency of the respirator. Prior to the experiments, the probes, 35 kD, MAB 2.14 (Microbiotech AB) with a membrane length of 2 mm, were placed in an Eppendorf vial and perfused for 10 min with Krebs Ringer solution adjusted to pH 7.0. According to the atlas of Paxinos & Watson (1998) dual probes were implanted vertically in the left and right dorsal hippocampus at the coordinates 0.38 mm posterior to the bregma, 0.22 mm lateral to the midline, and 0.38 mm below the surface of the dura mater.

The probes were perfused with the Krebs Ringer solution described above at a constant rate of 1 µL/min and only used once. The perfusate from the first 45-60 min was discarded to reach a steady baseline for the concentration of galanin-LI (Consolo et al., 1994), and further collected in one hour intervals in cold tubes containing 2% ethylele

glycol-bis(β-aminoethyl ether)-n,n,n´,n´-tetraacetic acid (EGTA)/Glutathion (Sigma). The dialysate after the first hour represents baseline (100%). After one hour, the animals received a subcutaneous injection in the neck of 40 mg/kg 17β-estradiol in 30 µL sesame oil. Dialysate was collected at the baseline and at one and two hours after injection of 17β-estradiol or vehicle. The dialysates from the right and left HiFo were pooled into one vial for each period, immediately frozen in liquid nitrogen and stored at -70°C until analysis by RIA.

The location of the probe was verified in all animals by injecting cresyl-violet into the probe at the end of the experiment (Fig 4), slicing 1 mm thick and computer-scanning the slices. Two rats in the control group and one in the estrogen-treated group were excluded, since the probes in these cases were not optimally located.

Immunohistochemical studies

For immunohistochemistry, the rats were anesthetized, intubated, and perfused via the ascending aorta with Tyrode's Ca^{+2}-free solution at 37°C, followed by a mixture of 4% paraformaldehyde and 0.4% picric acid in 0.16 mol/L phosphate buffer (pH 6.9, 37°C) (Zamboni & De Martino 1967) and then by the same, but ice-cold mixture. The brains were rapidly dissected out, immersed in the same fixative for 90 min and rinsed overnight in 10% sucrose in 0.1 mol/L phosphate buffer (pH 7.4). For *in situ* hybridization, the rats were sacrificed by decapitation, and the brains were dissected out, briefly immersed in ice-cold phosphate-buffered saline, sliced and immediately frozen on dry ice.

Antisera against galanin(1-16)

Galanin(1-16) antisera were raised in New Zealand white female rabbits. One milligram synthetic rat galanin(1-16) (Neosystem, Strasbourg, France) was coupled to 4 mg bovine serum albumin (Sigma) with 20 mg carbodiimide in 0.3 mL 0.025 mol/L phosphate buffer (pH 7.4). The mixture was gently stirred for 24 hours at 4º C, and dialyzed against 2 L saline for 24 hours. The dialysate was emulsified in Freund's complete adjuvant (DIFCO, Laboratories, Detroit, USA). Each of 10 rabbits received a single-site subcutaneous injection containing 100 µg galanin(1-16). Three booster doses of the conjugate were administered in Freund's incomplete adjuvant at 5-6 weeks intervals.

Labelling of galanin(1-16), (1-29) and NPY(1-36) with ^{125}I

^{125}I - Labelling

Synthetic galanin(1-16), (1-29) and NPY(1-36) (Neosystem) were labelled with radioactive iodine (^{125}I), using the chloramine-T method (Greenwood et al., 1963). The peptides (10 µg) were then dissolved in 10 µL each of 0.25 mol/L phosphate buffer (pH 7.4), and subsequently 1 mCi of ^{125}I was added (Amersham, Pharmacia, Biotech, Sweden). Fifteen µg of chloramine-T in 0.25 mol/L phosphate buffer (pH 7.4) were added to the mixture. The peptide, ^{125}I and buffer cocktail were then mixed continuously for 15-20 sec. Finally, the reaction was stopped with 15 µg sodium bisulfite (Sigma) in 10 µL 0.25 mol/L phosphate buffer. After mixing, 100 µL phosphate buffer and 100 µL 0.5% BSA in phosphate buffer were added.

Purification

The reaction mixtures were purified by reverse-phase high pressure liquid chromatography (HPLC) using a Nucleosil C_{18}, 5 µm 4.6 x 300 mm column (Merck) and eluted (1 mL/min) with a 40 min linear gradient of 20-50% acetonitrile in water containing 0.1% trifluoroacetic acid. Fractions of 1 mL were collected. The specific activity of the radioligand was about 70 Bq/fmol as determined by self- displacement.

Extraction of tissue and plasma samples

Tissues

The tissues were cut into small pieces on ice, and 10 mL of 1 mol/L acetic acid (Merck) were added per gram tissue and boiled for 10 min. The tissues were homogenized with a polytron CAT X520D (Zipperer, Staufen, Germany) and centrifuged at 1,500 x g in 4°C for 10 min. Immediately after collection of the supernatants, a second extraction was performed with 10 mL of distilled water per gram tissue. The supernatants from each sample were combined, lyophilized and stored at –70ºC. All samples were extracted and analyzed in randomized order.

Plasma

Two mL diethyl ether was added to 200 µL plasma in glass tubes and vortex-mixed for 30 sec. The tubes were subsequently frozen in 95% ethanol containing dry ice. When the aqueous fraction was frozen, the supernatant was decanted into another glass tube. The ether was evaporated at 40ºC. The extracted samples were dissolved in 0.05 mol/L phosphate buffer, pH 7.4, containing 0.2% BSA (Sigma) and 0.1% Triton X-100, and kept at 40ºC for 30 min before vortexing and cooling to room temperature.

Radioimmunoassay

Galanin and NPY in tissue extracts

The lyophilized samples were reconstituted in 1 mL of phosphate buffer (0.05 mol/L, pH 7.4), and 100 µL of each sample, antiserum and calibrator were used. The concentrations of galanin- and NPY-LI were measured using, respectively, a rabbit anti-galanin(1-29) antiserum (GAL4) (Theodorsson & Rugarn, 2000), a rabbit anti-galanin(1-16) rat antiserum (**paper IV**) and a rabbit anti-porcine NPY antiserum (Theodorsson-Norheim et al., 1985). HPLC–purified, rat ^{125}I galanin was used as radioligand. Rat galanin(1-29), rat galanin(1-16) and rat NPY(1-36) were used as calibrators (Neosystem). All samples were analyzed in randomized order. Detection limit was 7.8 pmol/L.

Galanin in microdialysis samples

The microdialysis samples were concentrated (3.2- times) by lyophilizing 80 µL samples and dissolving them in 25µL phosphate buffer (0.05 mol/L, pH 7.4). The detection limit of the galanin RIA (Theodorsson & Rugarn 2000) was improved by using a total incubation volume of 75µL (25µL of each sample/standard, antiserum and radioligand), 1,000 cpm of radioligand and amount of antiserum sufficient for 30% binding. Sample/standard and antibody (GAL4) were pre- incubated (in carefully closed vials) for 48h and the radioligand subsequently added and incubated for further 18 hours.

Bound and free radioligand were separated using 50 µL SacSel (IDS, Boldon, UK).

After 30 min incubation in room temperature and 10 min of centrifugation at 2,500g, the bound fractions were counted on a GammaMaster for 10 min/vial, and the detection limit was 3.9 pmol/L.

17β-estradiol in plasma

17β-estradiol was analyzed using a commercially available radioimmunoassay kit (Estradiol Double Antibody kit KE2D; Diagnostic Products Co, Los Angeles, CA, USA), and the detection limit was 0.02 nmol/L.

Samples from tissue extracts, plasma extracts and microdialysis *in vitro*- and *in vivo*-release samples and calibrators were measured on a GammaMaster 1277 (LKB Wallac, Turku, Finland).

Chromatography

High performance liquid chromatography

Reversed-phase HPLC was performed by elution with a linear gradient of acetonitrile (20-50%) in water containing 0.1% trifluoroacetic acid. Samples were passed through Millipore GS filters (0.22 µm) prior to the chromatography, and 200 µL were then injected. Fractions (0.5 mL) were collected at an elution rate of 1.0 mL/min Each fraction was lyophilized and re-dissolved in 100 µL of 0.05 mol/L phosphate buffer, pH 7.4, containing 0.2% BSA before analysis. The fractions were assayed for immunoreactivity with RIA in the tubes used for their collection.

Gel-permeation chromatography

Gel-permeation chromatography was performed using Superdex Peptide HR 10/30 column (10 x 300 mm) (Amersham) eluted with 30% acetonitrile in distilled water containing 0.1% trifluoroacetic acid. A Pharmacia P-500 FPLC pump provided an elution rate of 0.5 mL/min Fractions of 1 mL were collected and lyophilized before analysis by RIA.

Histochemistry

Immunohistochemistry

The formalin-picric acid fixed brains were snap-frozen using CO_2. Fourteen-µm-thick coronal brain sections were cut on a cryostat (Microm, Heidelberg, Germany), and thaw-mounted on to chrome alum-gelatin-coated object slides. The tyramide signal amplification immunohistochemical technique (Adams 1992) was applied using commercial kit, employing the same rabbit polyclonal antisera raised against galanin(1-29) (GAL4) and galanin(1-16) (K2), respectively, as used in the RIA (Theodorsson and Rugarn 2000; **Paper IV**). Incubation with primary antiserum GAL4 (1:4,000-1.000.000) and K2 (1:5,000 - 1:40,000) overnight at 4°C was followed by horseradish-peroxidase-conjugated, swine anti-rabbit IgG (1:100, Dako A/S, Copenhagen, Denmark) and incubations according to the TSA-Plus Fluorescein System (DuPont, New England Nuclear, Boston, MA, USA). The specificity of antibodies was tested by preadsorption tests with an excess (10^{-6} or 10^{-5} mol/L) of galanin or galanin(1-16), respectively (Bachem, Bissendorf, Switzerland). Sections were mounted in a mixture of glycerol and 0.1 mol/L phosphate buffered saline (3:1), pH 7.4, containing 0.1% para-phenylenediamine (Sig-

ma) as anti-fading agent (Platt and Michael, 1983). After processing, the sections were examined in a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

In-situ hybridization

The frozen brains were cut at 14 µm thickness using a cryostat (Microm) and thaw-mounted onto "Probe On" slides (Fisher Scientific, Pittsburgh, PA, USA). Antisense oligoprobes complementary to nucleotides 152-199 of galanin mRNA (Vrontakis et al., 1987) and to nucleotides 546-586 of NPY (Eva et al., 1990) were synthesized by CyberGene AB (Huddinge, Sweden). The oligonucleotides were labeled at the 3' end using terminal deoxynucleotidyltransferase (Amersham, Buckinghamshire, UK) with [^{33}P] dATP (Du Pont-NEN) to a specific activity of 1-4 x 10^6 cpm/ng oligonucleotide. The oligoprobe was purified through ProbeQuant G-50 Micro Columns (Amersham). Sections were hybridized as described previously (Schalling et al., 1988; Dagerlind et al., 1992). Briefly, air dried sections were incubated in a hybridization buffer [50% formamide, 4xSSC, 1xDenhardt's solution (1% sarcosyl, 0.02 mol/L phosphate buffer, 10% dextran sulfate), 500 µg/mL heat-denatured salmon sperm DNA and 1x10^7 cpm/mL of the labeled probe] in a humidified chamber for 16-18 hours at 42^{0}C. After hybridization the sections were washed in 1xSSC at 55°C for 4x15 min and for 30 min at room temperature, then air-dried and dipped into Kodak NTB 2 emulsion (Kodak, Rochester, NY, USA) diluted 1:1 with water. After exposure at 4°C for 2-6 days, the slides were developed in Kodak D19, fixed in Kodak Unifix and mounted in glycerol-phosphate buffer. For specificity control adjacent sections were incubated with an excess (x100) of unlabelled probe.

A total of four sections from a series through the rostro-caudal extension of the LC was examined using a Nikon Eclipse E600 fluorescence microscope equipped with a dark-field condenser. Digital images acquired with Nikon DXM1200 digital still camera (using a x20 objective) were analyzed for mRNA levels in Scion Image 4.0 (Bethseda, MD, USA). Each captured image was calibrated to 256 pixel grey values (0 = white and 256 = black), and the mean pixel density was measured. The background levels were measured in separate images from outside of the section area. We assumed that silver grain density overlying neurons correlates directly with their level of mRNA expression. To estimate silver grain density, the mean pixel density was converted into relative optic density (ROD) using formula – log (256-grey value)/256). The background ROD level was calculated in the same way and was ultimately subtracted from the neuronal ROD. Data were expressed as a percentage of mean ROD relative to the control group equal 100% ± SD. The levels of galanin mRNA were examined in the rat LC, and NPY mRNA lebels were analyzed in several regions of the mouse brain (HiFo, caudate nucleus, cingulate cortex).

Results and Discussion

The main objective of the present thesis was to investigate, if 17β-estradiol treatment induces *short-term* (after 1 hour) effects on the levels of the neuropeptides galanin and NPY in brain areas not directly involved in reproduction. Biochemical (*RIA, chromatography, microdialysis)*, anatomical (*immunohistochemistry)* and molecular biology (*in situ hybridization)* methods were used for studies in rats and mice.

The most notable finding was that the concentrations of galanin- and NPY-LI can be *rapidly* altered by 17β-estradiol treatment in brain areas important for memory and mood, including the HiFo and cortex of rats and mice (**papers I** and **III**). The effects of 17β-estradiol on galanin in the HiFo were not only present in the model situation of ovx rats, but also at pro-estrous in rats with intact ovaries having normal estrous cycles. See Table 1 and 2 for a summary of previous results from our laboratory and results within the present thesis.

Our results indicate that the rapid effects of 17β-estradiol on galanin and NPY are not mediated through the 'slow' classical nuclear estrogen receptor pathway, but rather via a rapid membrane-related mechanism. The effect of 17β-estradiol appeared within one hour, which is a too short time period for the classic nuclear mechanism of the 17β-estradiol effects, and it was not affected by Tamoxifen® which is known to block the effects of 17β-estradiol treatment via classical nuclear receptors in the HiFo. Furthermore, the effects of 17β-estradiol treatment were dose- and duration- dependent (**paper II** and **III**). The rapid effects of 17β-estradiol on neuropeptide levels are likely to contribute to the fine-tuning of the target neurons, e.g. by modulation of the release and/or effects of classical neurotransmitters, including catecholamines, ACh, 5HT and amino acids.

Most published studies on the effects of estrogen mediated through membrane-related mechanisms have been performed *in vitro* using cell cultures (see Toran-Allerand et al., 2005). In these models the term 'rapid' or 'short-term' effects are used to refer to effects taking place on a time-scale from milliseconds to minutes, whereas 'long-term' effects are those that occur after hours to days. Our studies were performed in brain tissue of *intact* animals, where 17β-estradiol needs to be absorbed from the site of injection, transported to effector sites before acting on receptor elements on the effector cells. The time scale for the detection of the effects of 17β-estradiol in intact brain tissues is therefore naturally longer. The terminology used in the present thesis is consequently the following: *short-term* (1 hour or less) and *long-term* (more than 1-2 hours), since we use it to describe the time-scale of *changes* in neuropeptide levels in intact brains after systemic administration of 17β-estradiol.

Neuropeptides usually belong to families of related peptides having similar chemical structures and biological effects. The biological effects of galanin are mediated by the N-terminal end of the molecule, and no other naturally occurring molecules have been shown to contain this structure except GALP. Therefore we immunized rabbits with a conjugate containing galanin(1-16) and were fortunate to raise an antiserum specific for the C-terminal end of galanin(1-16). Using a RIA based on this antiserum we found a component in the female rat brain consisting of approximately 8 amino acids (**paper IV**). This putative galanin homologue was found in the same nerve fibers in the rat

brain as galanin(1-29) and responded to 17β-estradiol in a similar manner. However, the component found has lipophilic properties and a Stokes radius distinctly different from that of galanin(1-29). Therefore we hypothesize that the component may be a novel galanin(1-29) homologue with the same steric properties as the C-terminal end of the first 16 amino acids.

Long term effects of 17β-estradiol

In **papers II** and **III** the dose-duration relationships of the effects of 17β-estradiol on hippocampal galanin and NPY levels were investigated in ovx and naïve female rats and mice. Galanin-LI was analysed in extracts of brain tissues in response to three treatment periods with two different doses of 17β-estradiol. In rats the doses were chosen to achieve a state of high physiological exposure (near-term pregnancy) as well as a pharmacological concentrations. Only high physiological doses were used in mice. In rats we found that that 17β-estradiol induces a significant effect of dose ($p<0.001$) and duration ($p<0.001$) on galanin-LI concentration in the rat HiFo, but found no significant difference between the ventral and dorsal region (**paper II**).

In contrast to the results in rats, we found no evidence of effects on galanin- or NPY-LI in the HiFo of mice after 14 days of treatment (**paper III**). However, in both rats and mice there was a significant increase of galanin-LI in the pituitary gland ($p<0.001$). The responsiveness of galanin to changes in estrogen concentrations has been shown earlier in the rat (Vrontakis et al., 1987; Kaplan et al., 1988) and mouse (Shen et al., 1999) pituitary. The *long-term* effects of 17β-estradiol (weeks) on neuropeptide levels described above in both rats and mice are likely to be mediated through the classical ERα or ERβ.

Short-term effects of 17β-estradiol

Papers I & **III** demonstrate that already one hour of treatment with 17β-estradiol alters the concentrations of galanin- and NPY-LI in the brain of young adult ovx rats and mice and the concentrations of NPY transcript in the HiFo and striatum (caudate nucleus) of mice. In the female rat, galanin-LI increased in the HiFo (**paper I**), but we found no evidence of changes in the other brain regions studied. The rapid onset and the lack of effect of Tamoxifen® indicate that these effects may be mediated through mechanisms initiated by ERs in the plasma membrane.

We hypothesized that the rapid increase was at least partly due to a decreased release of galanin into the extracellular fluid, resulting in accumulation of galanin within the NA terminals in the HiFo and thus to an increase in its tissue concentrations. Microdialysis of ECF in the dorsal HiFo of nine ovx 17β-estradiol-treated rats (**paper I**) supported the hypothesis showing that the concentration of galanin-LI significantly ($p<0.001$) decreased (36.9%) at 2 h – when compared to eight control rats – after treatment with a single injection of 17β-estradiol.

Microdialysis – apart from being expensive and time-consuming – can be technically challenging, since neuropeptides including galanin tend to adhere to the microdialysis membrane e.g. by van der Vaals forces, possibly resulting in confounding changes in the measured concentrations of neuropeptides in the extracellular fluid due to the probe

itself. Therefore an experimental protochol using a control group is crucial. Our results, including such a control group, indicate that the decrease in the extracellular concentrations of galanin-LI in response to 17β-estradiol is due to decreased release of galanin from the nerve terminals and not the confounding result of galanin being adsorbed to the microdiaysis membrane.

In order to elucidate the physiological role of the 17β-estradiol-induced increase in galanin concentrations found in ovx rats, we studied the changes in galanin concentrations in the HiFo in 30 female rats during the normal fluctuations of 17β-estradiol during regular estrous cycles. Compared to the diestrous phase, the levels of galanin-LI increased (125%) significantly ($p<0.05$) in the proestrous phase (**paper I**), perhaps also indicating a decresed release and thereby increase in intracellular concentrations. Thus galanin levels in HiFo also change in rats with intact ovaries during the natural variations in 17β-estradiol concentrations.

Rat estrous cycles are in the order of 4 days and plasma estrogen concentrations change over hours and days. The effects of 17β-estradiol on galanin concentrations are therefore most likely to be exerted both by rapid signalling systems through cell membrane estrogen receptors and through the classical nuclear ERα- or ERβ-dependent mechanisms – related to the time frame of the estrogen effects.

In the HiFo, striatum and frontal cortex of ovx **mice**, NPY-LI *decreased* (44% ($p<0.001$) in the HiFo, to 59 % ($p<0.05$) in the striatum, to 75% ($p<0.05$) in the cingulate cortex (Fig. 1) and to 53% ($p<0.001$) in the frontal cortex (**paper III**), 1 h after a single injection of 17β-estradiol, hypothetically reflecting an increased release of NPY and a subsequent compensatory increase in NPY mRNA - shown with the same protocol after two hours treatment (**paper III**). The increase in the NPY transcript was found in the HiFo and striatum, but was not evident in the cingulate cortex. In the HiFo, these effects are likely to occur mainly in GABA interneurons (see Freund & Buzsaki, 1996).

The effects of 17β-estradiol on the brain have shown to be dose-dependent (MacLusky et al., 2005). Our observations that the lower dose (2.5 μg) of 17β-estradiol administered to ovx mice induced a more profound change in NPY-LI after one hour compared to the higher dose (5 μg) lend further support to this finding.

Species differences

The species difference between rats (Sprague Dawley) and mice (Balb/C) demonstrated in the present thesis (**papers I** and **III**) is possibly due to difference in the distribution of ERs in the brain (Shughrue et al., 1997 a,b Sheng et al., 2004). Both ERα and ERβ mRNA have been found in similar abundance in the HiFo and LC of ovx rats when compared to mice (Shughrue et al., 1997; Österlund et al., 1998). However, ERβ is abundant in the DRN of female and male mice, where Sheng et al. (2004) found no expression of the receptor in rats. In the 5HT galanin system, species differences between rats and mice have also been reported (Larm et al., 2003)

However, currently available data on the distribution of estrogen receptors even within the same species seems to be controversial. Immunohistochemistry showed immunoreactivity for ERβ in the LC (Mitra et al., 2003), whereas Vanderhorst and co-workers

(2005) found no evidence for the presence of ERβ-LI in the LC area, using another antibody. Anyhow, perhaps there are differences in the distribution of ERs between these two species that are related to the different effects on galanin-LI in the HiFo and cortex. Interestingly, species differences, when studying effects of 17β-estradiol on HiFo morphology, have been demonstrated by others comparing Sprague Dawley rats and Balb/C mice (Li et al., 2004). They showed that the estrogen-induced increase in spine density observed in the HiFo of female rats (Frankfurt et al., 1990; Wolley & McEwen 1992) could not found in the Balb/c mice or in several other mice strains. The doses used in our mice protocol were similar (2.5 and 5 μg) to those used in the study by Li and coworkers (2004) (1 and 5 μg).

Effects of 17β-estradiol on neuropeptide release

The mechanisms of release of neuropeptides from LDCVs in the brain are beginning to be elucidated in detail (see Hilfiker et al., 1999; Merighi 2002). However, at present we can only speculate as to the detailed direct and/or indirect mechanisms of the action of 17β-estradiol on the release of neuropeptides.

Molecules known to be crucial for exocytosis of neuropeptides in presynaptic nerve terminals (see Jahn & Südhof, 1994) are: 1) *syntaxin*, a pre-synaptic terminal protein, important for vesicle-plasma membrane docking processes (Bennett et al., 1993); 2) *synaptophysin* – important for vesicle fusion system; 3) synaptotagmin – interacts with plasmamembrane proteins (e.g. syntaxin) (Jahn et al., 1985; see Südhof & Rizo, 1996); 4) *Ca^{+2}-dependent activator protein for secretion* (CAPS) are neural/endocrine specific proteins (Walent et al., 1992; Ann 1997), present on LDCVs but not on SSVs (Berwin et al., 1998) and essential for LDCV secretion. In the brain, 17β-estradiol has been shown to alter the concentrations of several synaptic proteins important for regulated vesicle trafficking and exocytosis (Brake et al., 2001; Li et al., 2004; Ohtani-Kaneko et al., 2004; Lee et al., 2004). The presence of estrogen receptors in nerve terminals (Milner et al., 2001, 2004) as well as the presence of other novel estrogen-related membrane receptors (see Toran-Allerand et al., 2005) may mediate the *short-term* actions on neuropeptide release reported in **paper I**, and possibly those reported in **paper III,** but the detailed mechanisms remain to be further elucidated.

The frequency dependence of the release of neuropeptides is well established in the peripheral nervous system (Lundberg 1981, 1996; Lundberg et al. 1982, 1983, 1986). Analogous direct experimental evidence for the dependence of neuropeptide release on stimulus frequency in the CNS is less well elucidated. However, interneurons in the HiFo fire at high frequency after a seizure (Lacaille et al., 1990) in response to ACh (Pitler & Alger, 1992) and NA (CA1 interneurons) (Bergles et al., 1996), and NPY has been suggested to be released spontaneously from mossy fibers in the DG (Tu et al., 2005). Estrogen increases hippocampal excitability in hippocampal CA1 neurons (see Woolley, 2000) and enhances LTP (Foy et al., 2001; Kim et al., 2002). It is possible that the increase of estrogen and the subsequent increase in excitability in the HiFo enable neuropeptide release. This – hypothetically – might then result in different patterns and extra-cellular concentrations of neuropeptides during the pro-estrous phase. Both galanin and NPY are potent inhibitors of excitability in the HiFo through inhibition of glutamate release (see Mazarati 2004). The effects of estrogen on the release could be mediated

through the classical estrogen receptors, recently found in pre-synaptic membranes in the rat brain (Milner et al., 2001, 2004) or through novel membrane- related receptor(s) (see Toran-Allerand et al., 2005).

In mice there was a significant increase (153%) ($p<0.001$) of galanin-LI in the medulla oblongata, including the pons (**paper III**). Since many galanin-positive cell bodies are located in the LC it is possible that this increase mirrors an effect on the LC neurons, although this is a rather speculative explanation. Interestingly and on the contrary, NPY-LI was almost abolished in the this area of the same mice.

The rapid, 17β-estradiol-induced increase of galanin-LI in the HiFo of female rat was not blocked by the selective estrogen receptor modulator (SERM) Tamoxifen®, suggesting a membrane related effect rather than an effect on the genome. However, Tamoxifen® is not a specific antagonist, since it is known to exert agonistic effects in non-neuronal cells as well (Peach et al., 1997). However, the same dose of Tamoxifen® as was used in **paper III** has been shown to function as an antagonist in the female rat HiFo (Rudick et al., 2003). They showed an induction of c-Fos in hippocampal CA1 area, 2 h after acute injection of 10 µg of 17β-estradiol in 100 µg sesameoil (Rudick et al., 2000, 2003) (we used 15 µg in our rat protocol). This was followed by a decrease in c-Fos induction and a second increase after 24h. When administering Tamoxifen® before or at the same time as estrogen, the induction of c-Fos was totally blocked after 24h but not after 2h in the dorsal part of the HiFo.

As mentioned above, the concentration of NPY-LI decreased one hour after exposure to 17β-estradiol in the striatum (caudate - putamen). The effects of Tamoxifen® were not evaluated on this decrease. However, according to the machinery responsible for these rapid effects of 17β-estradiol on galanin and NPY in the brain of rodents, the effects in the striatum of mice are intriguing. Ever since 1973 the striatal tissue has been considered devoid of classical estrogen receptors (Pfaff & Keiner, 1973) and has served as a model "knock-out" area when investigating rapid non-genomic/indirect genomic effects. This paradigm is controversial, but it seems that in the adult rat there are very few of classical ERs of both types in the striatum (Shughrue et al., 1997 a,b). Rapid effects of estrogen and differences between genders have been reported in striatum and in dopaminergic functions, suggested to be mediated through a membrane-related mechanism (see Becker, 1999).

The mechanisms underlying our findings of rapid effects of 17β-estradiol in the HiFo remain to be investigated, e.g. how 17β-estradiol is functionally related to the suggested release of these two peptides and the estrogen membrane receptor protein involved. Also, simultaneous monitoring of GABA and NPY in the HiFo, of dopamine and NPY in the striatum and of NA and galanin in the rat would be valuable. Left for future investigations is also in which cells these events take place, and which galanin and NPY receptors are involved.

	Radioimmunoassay of galanin-LI in brain tissue extracts					
Brain region	**Pre-pubertal vs adult**	**Early pro-estrus**	**Ovx + 17β-estradiol short-term (hours)**		**Ovx + 17β-estradiol, long-term (days-weeks)**	
			Rat	**Mouse**	**Rat**	**Mouse**
Hippocampal formation	increase	increase	increase	no effect	increase	no effect
Frontal cortex	increase	*	no effect	no effect	increase	no effect
Striatum	no effect	*	no effect	no effect	no effect	no effect
Hypothalamus	no effect	*	no effect	no effect	no effect	no effect
Medulla oblongata pons	*	*	no effect	increase	*	no effect
Pituitary	increase	*	no effect	no effect	increase	increase

* Not investigated

Table 1: Summary of the results of galanin analyses in the experimental paradigms of the present thesis

	Radioimmunoassay of NPY-LI in brain tissue extracts					
Brain region	**Pre-pubertal vs adult**	**Early pro-estrus**	**Ovx + 17β-estradiol short-term (hours)**		**Ovx + 17β-estradiol, long-term (days-weeks)**	
			Rat	**Mouse**	**Rat**	**Mouse**
Hippocampal formation	no effect	no effect	NS effect (decrease)	decrease	*	no effect
Frontal cortex	no effect	*	NS effect (decrease)	decrease	*	increase
Striatum	no effect	*	NS effect (decrease)	decrease	*	no effect
Hypothalamus	no effect	*	no effect	no effect	*	no effect
Medulla oblongata pons	*	*	no effect	decrease	*	no effect
Pituitary	increase	*	no effect	no effect	*	no effect

* Not investigated, NS, not significant

Table 2: Summary of the results of NPY analyses in the experimental paradigms of the present thesis

Putative galanin homologues

In addition to studies of 17β-estradiol and its effect on galanin(1-29) and NPY, we investigated the possible presence of homologues to N-terminal sequences of galanin by raising antisera in ten rabbits, using the C-terminal end of galanin(1-16) as hapten. We found (**paper IV**) the presence of an immunoreactive component, possibly a novel homologue to the N–terminal sequence of galanin. This component had a Stokes radius corresponding to a peptide of about eight amino acids and was apparently located in the same cell bodies and fibers as galanin(1-29) but with no overlap with the pattern previously shown for GALP. Importantly, chromatographic analysis (using Stokes radius and lipophilic properties) of the endogenous immunoreactive material revealed that it was different from galanin(1-16) and galanin(1-29). The concentration of this homologue increased in the HiFo in response to 17β-estradiol treatment of ovx rats after four hours but not after one hour. This is congruent with the results obtained in **paper I** except that the effect was not as rapid. However, OVX itself did not influence the concentration of the newly found component – in contrast to what has been shown for galanin(1-29) (Rugarn et al., 1999b). Our data support the existence of a novel endogenous galanin homologue with similar structure and biological effects as N-terminal sequences of galanin.

In electrophysiological studies Xu and co-workers (1999) showed a subpopulation of cells in the HiFo, responding selectively to galanin(1-15) but not to galanin(1-16) or galanin(1-29). In contrast neurons in the LC nucleus responded to galanin(1-29) and galanin(1-16) but not to galanin(1-15). Since areas like the dorsal HiFo had been shown to have only very few binding sites for the parent peptide galanin(1-29) (Skofitsch & Jacobowitz, 1986; Melander et al., 1988), these studies together indicated presence of a new type of galanin receptor selective for N-terminal galanin fragments.

Conclusions

- the levels of galanin-LI in the rat HiFo are influenced by a raise in the concentration of 17β-estradiol both after *short-* and *long-term* exposure.
- the *short-term* effect of 17β-estradiol on galanin-LI in the HiFo of female rats could not be blocked by Tamoxifen®, indicating an effect different from the classical transcriptional machinery of ERs.
- there are species differences between rats and mice, particularly with regards to the *long-term* effects of 17β-estradiol on galanin-LI.
- *Short-term* treatment with 17β-estradiol alters the concentration of galanin-LI in the ECF from the dorsal HiFo, indicating a decreased release from hippocampal nerve endings.
- Galanin-LI fluctuates during a normal estrous cycle - that is increased in tissue extracts in the pro-estrous phase.
- 17β-estradiol rapidly influences NPY transcript in the mouse HiFo and striatum (caudate nucleus-putamen), the latter an area most likely not expressing ERs - indicating a membrane-related effect.
- the higher dose (5 μg) of 17β-estradiol used in the mouse model did not affect the levels of NPY in the same manner as the lower dose (2.5 μg).
- A novel, estrogen-influenced approximately 8-aminoacid-long galanin homologue is present in the rat brain and gut, with steric properties similar to the C-terminal part of galanin(1-16).

Perspectives

Already four decades ago it became apparent that estrogens influence not only the reproductive organs, but also higher functional centers in the brain. An impressive body of evidence has since accumulated showing 17β-estradiol to be much more than a reproductive hormone (see Pfaff et al. 1983; McEwen 1987, 2002 a, b; Wang et al. 2002). Besides its well known action on the hypothalamus and other brain areas involved in reproduction, 17β-estradiol influences e. g. the widespread serotoninergic and catecholaminergic pathways of the brain in addition to the basal forebrain cholinergic system and the HiFo. It also affects cognition and mood and contributes to the maintainance of the structural and functional integrity of the CNS e.g. by regulating synapse turnover in the hippocampus during the 4- to 5-day estrous cycle of the female rat inducing the formation of new excitatory synapses through NMDA receptors (see McEwen, 2002 a).

The overwhelming majority of earlier cell culture and animal experimental studies indicate that 17β-estradiol exerts beneficial/neuroprotective effects on the brain. It was therefore hoped that 17β-estradiol administration could reduce the risk of dementias and other brain diseases, cardiovascular diseases, osteoporosis, etc. During the late 1990'ies and early 2000'ies physicians therefore increasingly prescribed estrogen not only short term for alleviating menopausal symptoms, but also long term in the hope of alleviating risks of disease. The results of the experimental studies have only recently been put to the test in large controlled epidemiological studies which show that estrogen treatment in postmenopausal women *increases* the risk of cardio- and cerebrovascular diseases and of breast cancer (Hulley et al. 1998; Viscoli et al. 2001; Rossouw et al. 2002). Given the observed increase in risks, it has been recommended that hormone replacement therapy be used only for management of menopausal symptoms and for as short a duration as possible (Cheung et al. 2004; Turgeon et al. 2004). 17β-estradiol appears to be a hormone which needs to be tightly regulated for normal tissue function and used with caution in the clinic.

The present thesis indicates that changes in the concentration of 17β-estradiol in females at menarche, during estrous cycles, at pregnancy and parturition and at menopause may influence brain functions partially by influencing the concentrations of the neuropeptides galanin and NPY. Altered concentrations of these two peptides in the extracellular space can e.g. modulate the biological functions of NA and serotonin which possibly contribute to the mood swings and cognitive effects common in periods of changes in the levels of 17β-estradiol in females. Decreased concentrations of galanin in the extracellular space in response to elevated concentrations of 17β-estradiol could lead to decreased galanin-induced inhibition of the biological effects excerted by NA neurons (see Xu et al., 2005) (see Fig 5).

Neuropeptides are commonly considered to be primarily modulators of other transmitters, acting slowly and mainly in extreme conditions of e.g. injury and other types of disease. Our results indicate that neuropeptides may participate in neurotransmission also under physiological conditions, e.g. when the concentrations of 17β-estradiol change. Thus in the present work changes in the concentration of sex hormones during a normal ovulatory cycle influenced galanin concentrations in the rat hippocampus.

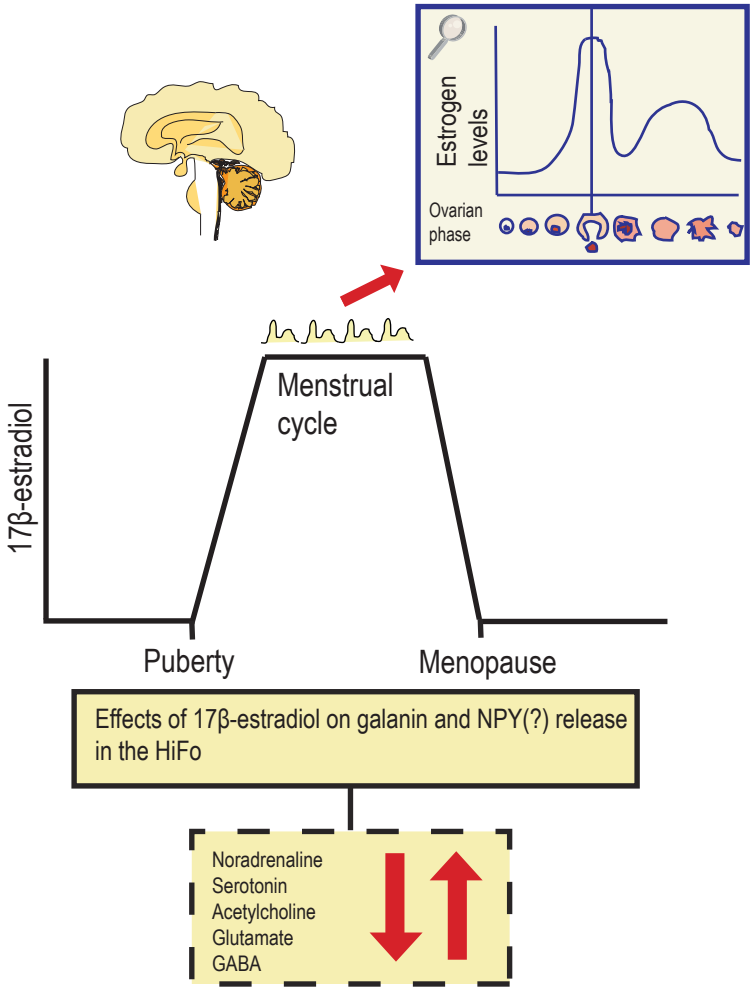

Figure 5

Dramatic and smaller changes in the levels of sex-hormones in general and of 17β-estradiol in particular influence cell- and bodily functions in females. Some of these effects may be mediated through the neuropetides galanin and NPY.

Several brain disorders in females seem to be related to the variation of sex hormones, including mood disorders (premenstrual syndrome, post-partum depression/ migraine) and epilepsy (e.g. catamenial epilepsy) (see Backström et al., 2003; Maguire et al., 2005). Both galanin and NPY are potent mediators of e.g. neuronal excitability and changes in their levels may be of physiological importance both in experimental animals and in humans.

In the present thesis, animal models were used in order to explore some aspects of the complex signalling systems of the intact brain. Since ovariectomy dramatically changes the overall hormonal homeostasis, we also investigated whether the same effect could be demonstrated during a normal ovarial cycle in intact rats. It turned out that galanin levels in the HiFo are also increased at proestrus in the normal cycle, though to a lesser extent than in the ovx rats subsquently treated with estrogen. The natural cyclical variation in the plasma concentrations of sex-hormones during the estrous cycle, in particular of 17β-estradiol, are known to influence excitability, dendritic spine outgrowth and the number of synapses in the HiFo (see McEwen 2000 a). Our data support that 17β-estradiol does not only exert its effects on the structural properties of the brain, but also on

the functional details in the neurochemical interactions between neurons.

Notably, it seems also that the effects of e.g. estrogen on the body depends on age (Sherwin, 2003, 2005). Therefore the results of the present thesis should be interpreted against the background that the rodents used in the studies were exclusively young.

It is also noteworty that there is considerable overlap between today's knowledge about estrogens effect on the rodent brain compared to those obtained in the human brain. Therefore the possibility exists that *short*-and *long term* exposure of the brain to 17β-estradiol – e.g. during a normal menstrual cycle or during pregnancy – have effects on galanin and NPY also in the human brain. This could be important for the regulation of acquisition, retention and mood. However, this requires to be shown. In fact the species differences between rat and mouse already shown here are reason for caution when extending our hypothesis to humans.

Memory

Memory studies in women generally indicate better performance during the luteal phase compared with the menstrual phase, suggesting that 17β-estradiol facilitates verbal and fine motor skills (Broverman et al., 1981). Functional magnetic resonance imaging studies in young women support the evidence of the constructive effects of estrogens on brain morphology reported in animals (Frankfurt et al., 1990, Woolley & McEwen, 1992) showing that neural recruitment in a cognitive task is highly responsive to fluctuations of gonadal steroid hormones (Fernández et al., 2003). These studies are in line with studies in rats using ^{125}I estrogen showing labelling to ERs in pyramidal cells of CA1 and CA3 in the ventral HiFo – areas important for memory (Shughrue & Merchenthaler, 2000), and early studies showing that estrogen increases the concentration of choline acetyltransferase, the synthetic enzyme for ACh (Luine et al., 1975), a neurotransmitter critically implicated in memory functions and which is markedly reduced in levels in AD (Davies & Maloney, 1976).

Galanin can be expressed in cholinergic forebrain neurons, that is co-localized with choline acetyltransferase (ChAT) in some species (Melander et al., 1985, 1986c) and inhibits ACh release in the HiFo (Fisone et al., 1987) which provides for a possible role of galanin in acquisition and retention (see Crawley, 1996; Ögren et al., 1999) and for a possible significance in AD in both animals and human (see Counts et al., 2003). Galanin impairs cognitive performance injected intraventricularly (Schött et al., 2000), while acting in an opposite direction when injected in the medial septum (Elvander et al., 2004). Taken together a rapid 17β-estradiol-induced decrease in extracellular galanin concentrations in the HiFo indicate that the effects of 17β-estradiol on galanin may participate in the facilitation of memory induced by estradiol at the level of the HiFo.

Mood & depression

Women (all ages) have been shown to suffer from a higher incidence of depression than men (Weissman et al., 1993). Clinical observations in women with premenstrual disorders or with major depression beginning in the post-partum period or during menopause point toward the importance of sex-hormones as neuroendocrine modulators of mood. Thus, estrogens have been suggested to decrease the susceptibility for depression (Halbreich et al., 1997). Dysfunctions in serotonin and NA transmission are implicated in depressive disorders. In rats estrogen increases the serotonin activity by improving the effect of serotonin reuptake inhibitors (see Birkhäuser 2002) and by affecting the serotonin receptors (Österlund et al., 1998). In the human brain, the ERα and ERβ mRNA are present both in the HiFo and LC (Österlund 2000) and ERβ has been found to be decreased in persons committing suicide (Östlund et al., 2003). Galanin-LI is expressed in a population of 5HT neurons in DRN and in NA neurons of the LC (Melander et al., 1986 b, c), and this peptide can affect the release of 5-HT and NA in the HiFo (Kehr et al., 2001; 2002). Hypothetically, a 17β-estradiol- induced decrease in galanin release in the HiFo (**paper I**) can affect both serotonin and NA transmission in the HiFo. NPY mRNA has been shown in the human brain, e.g. in the HiFo (DG) (Caberlotto et al., 1999 a, b), but data addressing a correlation between affective disorders and estrogen are limited.

Galanin homologue

Galanin – in contrast to NPY – is the only member of the galanin family with the exception for the recently discovered GALP. Normally neuropeptides belong to families of neuropeptides with similar structure and functions. Three galanin receptors have been cloned which only share amino acid identities in the order of 30-50%. Functional and radioligand binding studies indicate presence of galanin receptor subtypes in addition to the presently known receptors. However, there are no reports of endogenous galanin homologues with amino acids in the tree-dimensional conformation required for binding to the suggested additional galanin receptor(s). Another unique feature of galanin is that it exerts its biological effects by its N-terminal end while most other neuropeptides use the C-terminal end. Therefore we investigated a possible occurrence of homologues to the N-terminal part of galanin using an antiserum raised against galanin(1-16).

Since antibodies recognize three-dimensional structures of 3-5 amino acids in a peptide they may be able to recognize a similar three-dimensional structure in a related peptide, possibly an additional member of the galanin peptide family. We found a component consisting of about eight amino acids with different Stokes radius to that of galanin(1-29), galanin(1-16) but similar to galanin(9-16) and with different lipophilic characteristics compared to galanin(1-29), galanin(1-16) and to galanin(9-16). Furthermore, this homologue had similar distribution pattern in the brain as galanin(1-29) but with no overlap with the distribution pattern previously found for GALP. Moreover, the concentration of the new component increased in response to estrogen treatment in the same brain regions as shown for galanin(1-29). The homologue found may be a peptide structurally and functionally related to the galanin peptide family utilizing steric properties similar to that of galanin(1-16).

Electrophysiological studies (Xu et al., 1999) demonstrated a subpopulation of cells in the HiFo, which responded selectively to galanin(1-15) but not to galanin(1-16) or galanin(1-29). In contrast neurons in the LC responded to galanin(1-29) and galanin(1-16) but not to galanin(1-15). Since areas like the dorsal HiFo have been shown to have only very few binding sites for the parent peptide galanin(1-29) (Melander et al., 1988), and since the peptide is present in equal amounts in both dorsal and ventral HiFo (Skofitsch and Jacobowitz, 1986) these studies together indicate that a N-terminal galanin homologue may exert novel effects in the HiFo.

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