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THE ROLE OF ESTROGEN RECEPTORS IN THE AUDITORY SYSTEM

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ABSTRACT

Both laboratory and clinical studies have previously demonstrated estrogenic effects on auditory function. The overall scope of this study was to investigate the physiological and molecular involvement of estrogens and estrogen receptors alpha (ERα) and beta (ERβ) in hearing physiology. ERα and ERβ were localized in a number of central auditory structures in mice, and their differential localization suggested distinct roles in auditory processing. ER expression was assessed in young, prepubertal and aged mice with diverging levels of estrogens. Changes in the expression patterns were not uniform between groups, suggesting that region-specific mechanisms regulate ERs expression. Neither age group showed sex differences in ER expression. Chronic 17β-estradiol treatment in ovariectomized mice resulted in molecular changes in the central (inferior colliculus) and peripheral (cochlea) auditory structures. Down-regulation of ERα mRNA in the cochlea and inferior colliculus may be a direct effect of estrogen-induced feedback inhibition of ERα transcription. No changes were noted for ERβ mRNA levels, suggesting that ERβ is constitutively expressed, rather than directly regulated by circulating hormones. Concurrent with these molecular changes, auditory-related behavioral parameters were altered by 17β-estradiol treatment. Improved prepulse inhibition of the acoustic startle response after 17β-estradiol treatment, suggested an estrogenic modulation of sensorimotor gating. Investigation of mice deficient in ERα (ERKO mice), ERβ (BERKO mice) and aromatase (ARKO mice) suggested a protective role for ERβ in the auditory system against acoustic trauma. Brain derived neurotrophic factor (BDNF), which is a neuroprotective peptide that can be induced by estrogens, increased in the cochlea after treatment with an ERβ-selective agonist, whereas it was decreased in the cochlea of BERKO and ARKO mice. ERβ-mediated neuroprotective mechanisms against noise exposure involving neurotrophic factor BDNF, were suggestive of estrogens' supportive contributions to the auditory function. Analysis of ERα, ERβ and BDNF levels in the cochlea during the reproductive cycle, revealed regulation of ERα but not ERβ or BDNF by endocrine activity. ERα levels were lower in high-estrogen conditions, suggesting that ERα expression in the peripheral auditory system is regulated by circulating sex hormones and acts as an interface between endocrine activity and the auditory system. Taken together, these results suggest an involvement of estrogens and their receptors along with neurotrophic factors in the physiology of the mammalian auditory system. Unraveling the distinct roles of estrogen receptors in the auditory system may provide novel treatment strategies and pharmacological targets for the support of hearing.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to the text by their Roman numerals:

I. CHARITIDI K, Canlon B. Estrogen receptors in the central auditory system of male and female mice. *Neuroscience*. 2010 Feb 3;165(3):923-33.

II. CHARITIDI K, Frisina RD, Vasilyeva ON, Zhu X, Canlon B. Expression patterns of estrogen receptors in the central auditory system change in prepubertal and aged mice. *Neuroscience*. 2010 Nov 10;170(4):1270-81.

III. CHARITIDI K, Canlon B. Characterizing molecular and behavioral responses to estradiol manipulations in the auditory system. *Manuscript.*

IV. Meltser I, Tahera Y, Simpson E, Hultcrantz M, CHARITIDI K, Gustafsson JA, Canlon B. Estrogen receptor beta protects against acoustic trauma in mice. *J Clin Invest.* 2008 Apr;118(4):1563-70.

Additionally, the following preliminary results contribute to the scope of this thesis:

CHARITIDI K., Canlon B. Biochemical alterations in the auditory system during the estrous cycle. *Preliminary results.*

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LIST OF ABBREVIATIONS

ABR	auditory brainstem response
ARKO	aromatase knockout
ASR	acoustic startle response
AuC	auditory cortex
AuN	auditory nerve nucleus
BDNF	brain-derived neurotrophic factor
BERKO	ERβ knockout
BIC	brachium of the IC
CIC	central nucleus of the IC
CN	cochlear nucleus
DCIC	dorsal cortex of the IC
DCN	dorsal cochlear nucleus
DLL	dorsal nucleus of the lateral lemniscus
DPN	2,3-bis (4-hydroxyphenyl)-propionitrile
ECIC	external cortex of the IC
ERKO	ERα knockout
ERα	estrogen receptor α
ERβ	estrogen receptor β
IC	inferior colliculus
ILL	intermediate nucleus of the lateral lemniscus
LL	lateral lemniscus
LSO	lateral superior olive
LVPO	lateroventral periolivary nucleus
MGN	medial geniculate nucleus
MSO	medial superior olive
MVPO	medioventral periolivary nucleus
OVX	ovariectomy
PPI	prepulse inhibition
PPT	propyl (1H) pyrazole-1,3,5-triyl-trisphenol
SOC	superior olivary complex
SPO	superior paraolivary nucleus
Tz	nucleus of the trapezoid body
VCA	ventral cochlear nucleus, anterior part
VCN	ventral cochlear nucleus

VCP	ventral cochlear nucleus, posterior part
VLL	ventral nucleus of the lateral lemniscus

1 INTRODUCTION

The auditory system is a miraculous example of micromechanics, sound engineering, and a strictly controlled biological milieu. Several of its features such as tonotopic organization of frequencies, speed and sensitivity of transduction, sound amplification, ability to locate sources of sound in space, the hair cells' capacity to perceive motions of atomic dimensions and a wide range of frequencies, make it a uniquely sophisticated example of sound engineering. Damage or decline in the function of the cochlea or the central auditory system accounts for communicative disorders such as hearing disabilities and deafness, which affect millions of people. Like every other part of our body, the auditory system is not likely to exhibit a static function unaffected by what happens to the rest of the organism, but is rather under continuous control of the specific needs at every time-point by receiving input through hormones and neurotransmitters. Sensory receptors' excitability, neuronal transmission, ion homeostatic mechanisms and synaptic plasticity are likely modulated by signals reaching the auditory system from the rest of the body, thereby optimizing auditory function. However, little is known about the effects of estrogens on the auditory system.

The extensive interactions between gonadal hormones and the nervous system are well documented both during development and in adults. A handful of such manifestations include modulation of neuronal excitability by gonadal steroids both by directly affecting the neurotransmitter systems and by affecting intrinsic membrane properties (Joels, 1997), sex differentiation of the brain by exposure to gonadal hormones (Arnold and Gorski, 1984, McCarthy, 2010), estrogenic stimulation of neuronal and dendritic growth in several brain regions, such as the hypothalamus (Toran-Allerand, 1976) and the hippocampus (Gould et al., 1990), changes in synaptic regulation and plasticity over the reproductive cycle (Brussaard et al., 1999) or sex-steroid-related seasonal changes in the brain and the singing behavior in songbirds (Ball et al., 2002). What is it that it is known about estrogens' effects on the auditory system? How are they enacted in the auditory system? What is the contribution of each estrogen receptor type? Which levels of the auditory processing do they affect? Are these effects protective or diminishing? Would estrogen therapy have any impact on the auditory system? This thesis considers the auditory system and how it is influenced by estrogen, describes the distribution of estrogen receptors in the central and peripheral auditory system, investigates the effects of ovariectomy and chronic 17β-estradiol treatment on the expression of estrogen receptors in the auditory system and on auditory-related behavior, examines the effects of genetic ablation of estrogen receptors and aromatase in the auditory function, as well as the biological effects of hormonal fluctuations during the reproductive cycle on the auditory system, and outlines therapeutic possibilities for the protection from deafness.

1.1 AUDITORY SYSTEM AND HEARING LOSS

The cochlea is the auditory component of the inner ear, which is located in the temporal bone, and contains the sensory organ of hearing, namely the organ of Corti (Fig. 1). Sound waves, which are transmitted into the inner ear via the outer and middle ear amplifiers, displace the basilar membrane and the sensory cells. This causes the sensory receptors' stereocilia, which are attached to the tectorial membrane, to deflect causing the depolarization of the sensory receptors. There are two types of auditory receptors, the inner (IHCs) and outer hair (OHCs) cells. Hair cells are tonotopically arranged in rows along the length of the organ of Corti, with outer hair cells

outnumbering the inner hair cells by 3:1 rows. Inner hair cells are the main sensory receptors responsible for the transduction of sound waves to electrical signals to the brain, whereas outer hair cells act as amplifiers. Primary afferent neurons synapsing with IHCs have their cell bodies into the central part of cochlea called modiolus, where they form the spiral ganglion. These are known as type I spiral ganglion neurons (SGNs), are large myelinated neurons, comprise approximately 90% of the SGNs and the ratio of type I SGNs to IHCs innervations is 20:1. OHCs on the other hand are innervated by type II SGNs, which are smaller unmyelinated neurons with an inverse relation to OHCs innervations, approximately 1:20. The stria vascularis, in the lateral wall of membranous cochlea, is responsible for generating the endocochlear potential by draining Na^+ from endolymph and together with spiral ligament, which keeps ion balance by transporting K^+ from perilymph to endolymph they keep a stable ionic milieu in the cochlea.

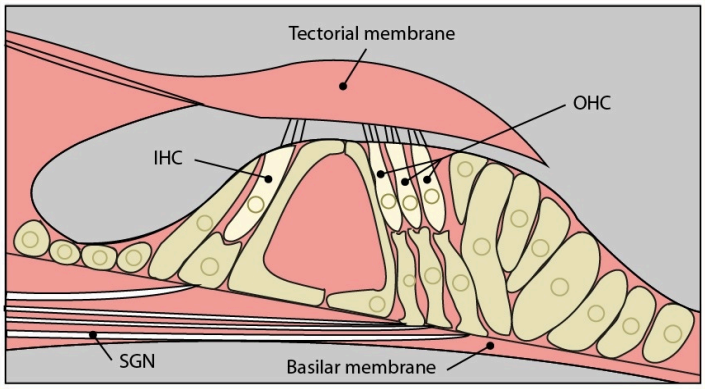

Figure 1. Cross section of the cochlea showing the organ of Corti. Sensory receptors are arranged in one row of inner hair cells (IHC) and three rows of outer hair cells (OHC) along the basilar. Spiral ganglion neurons (SGN) synapse with hair cells transmitting information to and from the brain.

Encoded sound information is transmitted via the auditory nerve from the cochlea to the central nervous system (Fig. 2). The auditory nerve enters the brainstem at the pons-medulla junction and its neurons synapse with neurons of the cochlear nucleus. From there, secondary auditory neurons give off axons, which decussate in the trapezoid body of ventral pons and ascend to the superior olivary complex of the contralateral side in the lower pons, which is important for detecting interaural level and time differences necessary for sound localization. Third order neurons of the superior olivary complex send axons via the lateral lemniscus to the inferior colliculus of the midbrain, which is an important level of binaural information processing and a major site of integration of auditory information. Fourth order neurons continue to the medial geniculate body, the thalamic relay where sensory information is filtered before it is further transmitted to the cortex. Axons of fifth order neurons of the medial geniculate body synapse with neurons of the auditory cortex on the superior temporal gyrus. This traditional view of the auditory system is completed with alternative routes, which have a substantial influence on auditory processing, like descending cortico-cochlear pathways, which send neural feedback to the periphery thereby dynamically controlling the flow of auditory information transmitted from the hair cells to the brain, projections to non-auditory structures and fibers that decussate at different levels or bypass certain auditory nuclei.

According to the World Health Organization (WHO) in 2005 about 278 million people had moderate to profound hearing loss. The heavy social and economic burden imposed on individuals, families, communities and countries, stresses the importance of prevention and correct management of hearing impairment. Hearing impairment is classified in conductive (outer and middle ear) and sensorineural (inner ear or auditory nerve) hearing loss. The most common causes of hearing loss according to WHO are

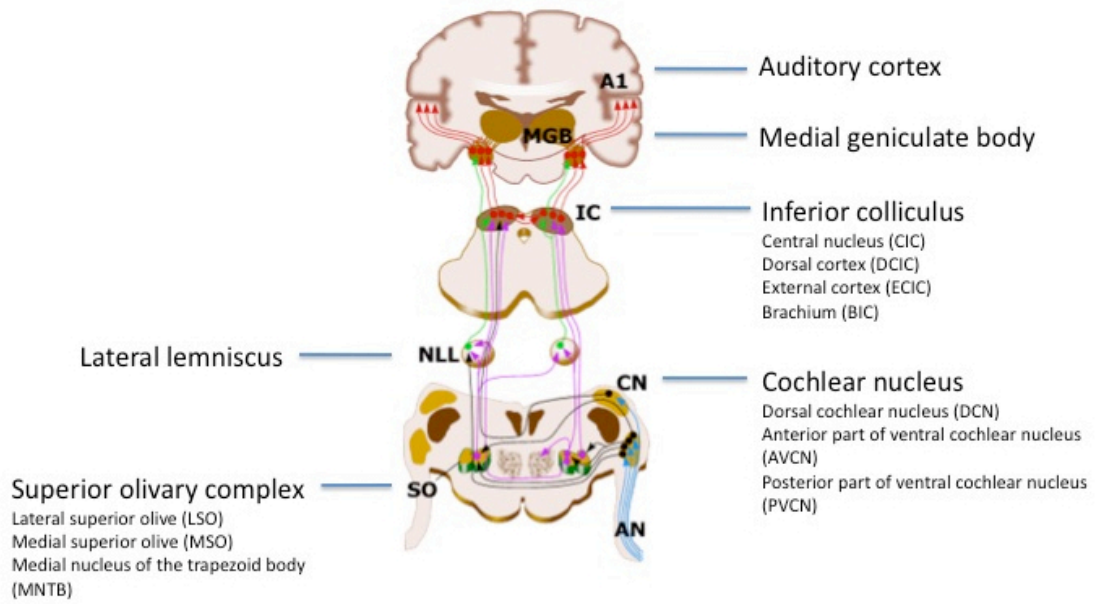

Figure 2. The central auditory system, with its main structures.

infectious diseases, noise exposure, head and ear injury, ageing and ototoxic drugs. In this work we will specifically refer to age-related hearing loss and noise exposure, as these are the main causes of sensorineural hearing loss. Forty per cent of the population over 65 years old have some degree of hearing impairment (Ries, 1994) and eighty per cent of hearing impairments affect the elderly population (Davis, 1990). Presbycusis is commonly divided into central and peripheral, depending on whether the central or peripheral components of the auditory pathway are mostly affected. Peripheral damage accounts for most cases of presbycusis and can be further divided to metabolic (stria atrophy), sensory (hair cell loss), neural (ganglion cell loss) and cochlear conductive (stiffness of basilar membrane) (Gates and Mills, 2005). Presbycusis or age-related hearing loss is regarded as the outcome of years of extrinsic auditory insults such as accumulated environmental noise toxicity, trauma, otological diseases and ototoxic substances on the intrinsic genetic substrate accompanied with the normal ageing process; with noise and ageing being the main factors (Gates and Mills, 2005). Age itself leads to gradual loss of hearing sensitivity, as shown in experiments where the animals were raised in a quiet environment without any exposure to noise or other ototoxic factors. On the other hand, sole exposure to excessive noise (without the age factor), including occupational noise, loud music or other loud noises such as gunfire or explosions, cause significant damage in the inner ear and lead to hearing impairment or deafness.

1.2 ESTROGENS

17β-estradiol (Fig. 3) is the most abundant circulating estrogen, synthesized primarily in the ovaries from cholesterol via aromatization of testosterone by the enzyme aromatase. Other tissues such as the liver, fat, adrenal glands and the brain produce estrogens in smaller amounts. Their evolutionary presence from mollusks like *Aplysia californica* (Thornton et al., 2003) to vertebrates classifies them among the most ancient forms of

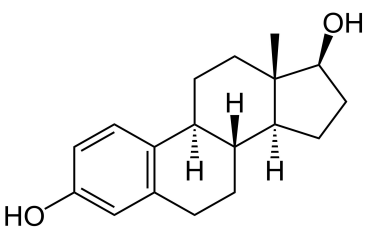

Figure 3. 17β-estradiol

intracellular communication. Estrogens are traditionally thought of as the female reproductive hormones and their levels vary through the estrous cycle, peaking before ovulation. Notwithstanding their role in sexual differentiation and reproductive function, their effects on functional processes in the brain of both males and females are well-established and include sensory and cognitive functions, to such a degree that they are considered to act in the brain like neurotransmitters or neuromodulators (Joels, 1997, Balthazart and Ball, 2006). Steroid regulation of neuronal excitability adds another aspect to the regulation of physiological processes in the brain and their behavioral outcome (Joels, 1997). As outlined in the latter review, estrogen-induced changes in neuronal excitability are achieved both through fast non-genomic pathways, which bring rapid changes in phosphorylation or in the electrical properties of neurons (modulating membrane resistance, ion conductivity and thus neuron excitability), and through delayed gene-mediated pathways (modulation of expression of neurotransmitter receptors, dendritic morphology). These estrogenic effects on neuronal firing are mostly excitatory, even though area-specific differences clearly occur, and are achieved conditional on the local neuronal network either by facilitating excitatory events or by suppressing inhibitory properties (Joels, 1997). Furthermore, estrogens improve the plasticity of neurons, dendrites and synapses (Brinton, 2009) and have neuroprotective effects against brain ischemia (Dubal et al., 1998, Suzuki et al., 2009).

Estrogens exert their actions through multiple strictly controlled pathways. Briefly, they act either through fast non-genomic pathways or through more delayed gene-mediated pathways. A summary of the pathways of estrogen actions can be seen

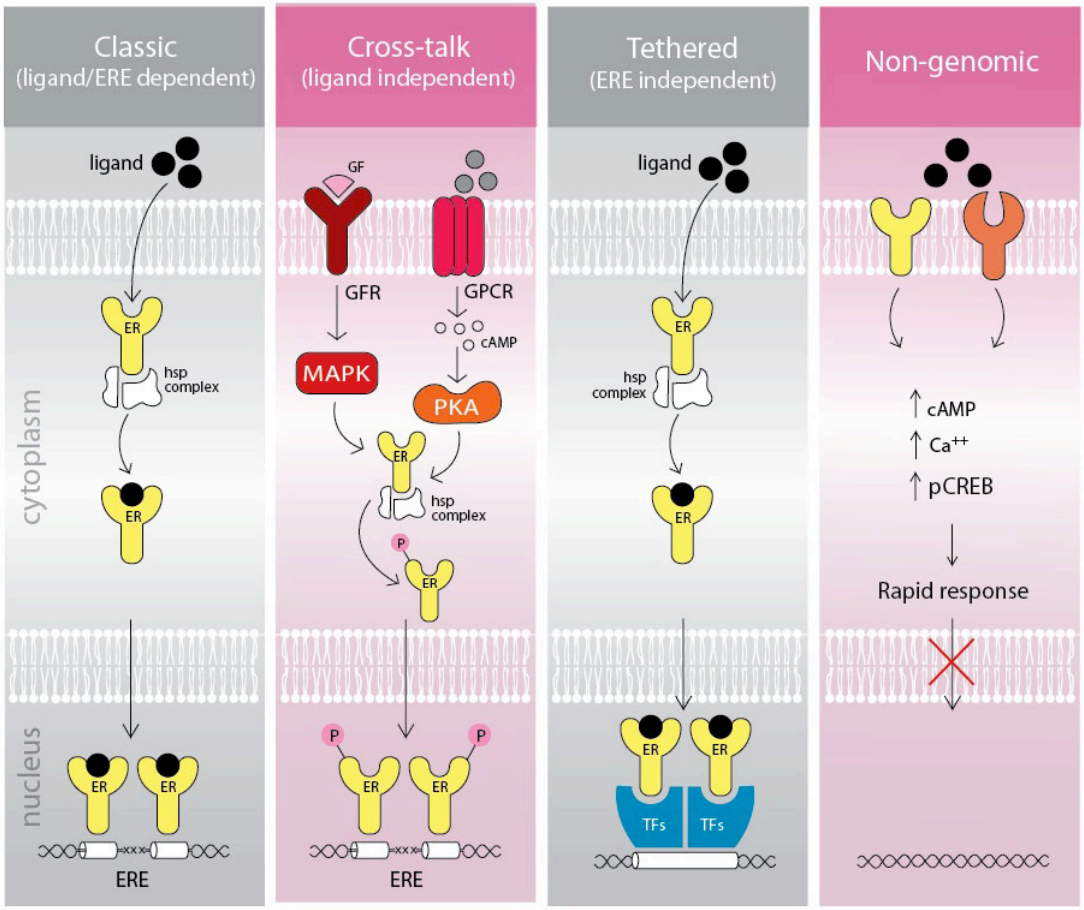

Figure 4. Mechanisms of estrogen signaling. MAPK: mitogen-activated protein kinase; PKA: protein kinase A. Reproduced with permission of Elsevier from (Charitidi et al., 2009).

in figure 4: (A) In the classic ligand/ERE dependent pathway estrogen receptors (ERs), which lie inactive in the heat-shock-protein (hsp) complex, are activated by a ligand, dimerize and bind to specific DNA sequences of the target genes called estrogen response elements (EREs), and up- or down-regulate their transcription. (B) In the absence of ligand, ERs are activated by other messengers, such as growth factors (GFs) or G-protein coupled receptors (GPCR), leading via second messengers to phosphorylation (P), dimerization and binding of ERs to their EREs. (C) Ligand-activated ERs 'tether' to other transcription factors (TFs) directly bound to DNA, instead of binding their own EREs, and thus acting more as co-regulators than transcription factors themselves. (D) ERs or other binding sites in the membrane induce second messengers, such as intracellular calcium, cAMP, or the phosphorylation of the cAMP response element binding protein (CREB), and elicit rapid cellular responses which do not require transcription of genes (Losel et al., 2003).

1.3 ESTROGEN RECEPTORS

ERs are nuclear receptors and act mainly as ligand-activated transcription factors. There are two types of ERs, alpha (ERα) and beta (ERβ), which are products of discrete genes (ESR1 and ESR2 respectively) and interact as complementary opposites, constituting parts of a complex dynamic system in the body (Lindberg et al., 2003). The human ERα cDNA was cloned for the first time in 1985 (Walter et al., 1985) and sequenced the following year (Green et al., 1986). Approximately a decade later the ERβ gene was found in the rat (Kuiper et al., 1996) and human (Mosselman et al., 1996). Since then, ESR1 and ESR2 have been isolated from a variety of species, including the mouse (White et al., 1987, Tremblay et al., 1997). In human, ESR1 is more than 140 kb long (Ponglikitmongkol et al., 1988) split into 8 exons and localized on chromosome 6q25.1 (Menasce et al., 1993), and its product (ERα) is a 595 amino acids protein with a molecular weight of circa 66 kDa. In mouse ESR1 was mapped on chromosome 10 (Justice et al., 1990) encoding a 599 amino acids protein with an overall homology of 88% in the sequence between the two species. In human, ESR2 was mapped on chromosome 14q and its product (ERβ) is a protein of approximately 60 kDa consisting of 530 amino acids, whereas in the mouse it has a molecular weight of 63 kDa and consists of 549 amino acids. Multiple promoters of ERα and ERβ have been identified in humans, rats, mice and other species. An example is a functional 46-kDa ERα isoform which is a product of alternative splicing and divergent promoter utilization (Flouriot et al., 2000). The existence of various promoters explains the presence of different mRNA variants and ER isoforms, as well as the differential expression of the receptors in different

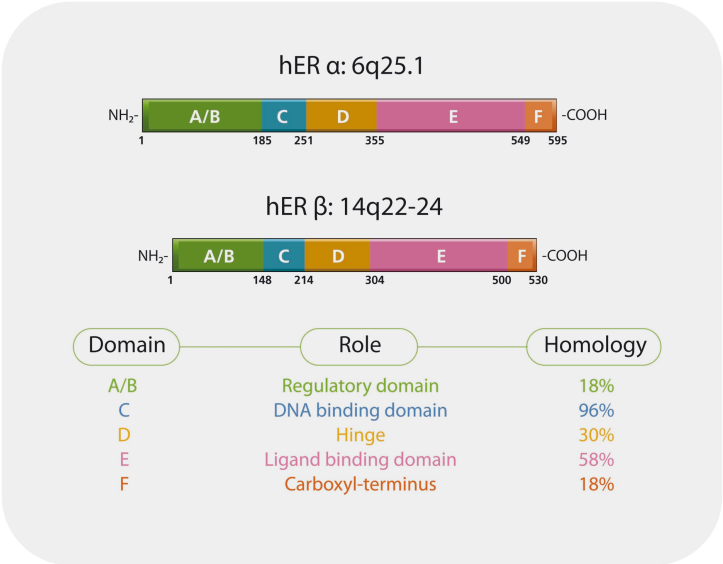

Figure 5. Functional domains of human estrogen receptors alpha and beta (hERα and hERβ). Reproduced with permission of Elsevier from (Charitidi et al., 2009)

tissues and cell types.

The sequence of ERα was divided into 5 functional units (A/B, C, D, E, F) (Fig. 5) based on the homology between human and chicken ER: domains C and E have a high degree of sequence homology (100% and 94%, respectively) and represent the DNA-binding domain and hormone-binding domain of the receptor respectively (Krust et al., 1986). Additionally, an extremely well conserved interspecies molecular mechanism of activation of the gene encoding ERα, was shown when human ER was expressed in yeast and apparently acted as an estrogen-dependent transcription factor (Metzger et al., 1988). The E domain, or ligand-binding domain (LBD), binds specifically to the ligand and activates ligand-dependent transcription. Even though ERα and ERβ LBDs vary substantially, they have similar affinity for 17β-estradiol, the main estrogen found in the body. The C domain, or DNA-binding domain, binds with high affinity to the EREs of estrogens' target genes. Comparison between the two receptors reveals an extremely well-conserved DNA-binding domain (96% homology) and a less conserved (58% homology) ligand-binding domain (Mosselman et al., 1996). Despite differences in their LBD, the two receptors have similar affinity for 17β-estradiol (Kuiper et al., 1997). The high variability in regulatory domains accounts for differential expression, distinct regulation and often opposing functions between the two ER types (Pearce and Jordan, 2004). Examples include the differential expression of ERs in the ovaries, where ERα is expressed exclusively in the theca and interstitial cells, whereas ERβ is found solely in the granulose cells (Fitzpatrick et al., 1999), a microarray analysis revealing that ERβ reduces 85% of ERα-regulated transcription of genes in liver and bone (Lindberg et al., 2003) and the well-established opposite effects of ERα and ERβ on cell proliferation and apoptosis (Thomas and Gustafsson, 2011).

1.4 ESTROGENS AND HEARING

1.4.1 Laboratory evidence

A number of studies suggest that the physiological and behavioral output of sensory systems including the auditory system, is modulated by estrogens in many species (Maney and Pinaud, 2011). Locally synthesized estradiol rapidly affects responses to auditory stimuli via interaction with neurotransmitters in the auditory cortical analog in songbirds (Tremere et al., 2009). In the auditory caudo-medial nidopallium (NCM, corresponding to the mammalian auditory cortex) of zebra finch, local estradiol and testosterone levels are differentially regulated by glutamate and GABA respectively, thereby rapidly modulating auditory processing in the songbird forebrain (Remage-Healey et al., 2008). The interplay between gonadal hormones and neurotransmitters is further supported by evidence from the auditory system of marine vertebrates. 17β-estradiol and testosterone refine auditory sensitivity of ovariectomized midshipman fish, by increasing the temporal encoding of male vocalization frequencies in the inner ear (Sisneros et al., 2004). Such steroid-dependent plasticity of the auditory sensitivity improves vocal-acoustic communication in midshipman fish (Sisneros, 2009). Beyond the receptive and neural components of the auditory system, estrogens also affect the homeostasis of the ions in the cochlea through direct non-genomic actions on stria vascularis. Non-genomic effects of 17β-estradiol in the stria vascularis include the inhibition of ion transportation through the gerbil stria vascularis *in vitro* (Lee and Marcus, 2001), resulting in the alteration of the strictly controlled milieu in the cochlea and potentially the mechano-electrical transduction of hair cells.

In addition to these fast non-genomic actions, estrogen-induced gene transcription modulates auditory processing, mainly via transcription factors ERα and ERβ. ERs are located in the cochlea of mouse and rat, indicating that estrogens affect sensory processing already from the auditory periphery (Stenberg et al., 1999). ERs

expression in the cochleae of mice is affected by sex and age (Motohashi et al., 2010) and it varies with the hormonal milieu in the inner ear of fish (Maruska and Fernald, 2010). ERs are down-regulated by high estrogen levels, as shown in a study examining ERs expression in the cochlea of rats in different stages of maturation and pregnancy (Simonoska et al., 2009a). Another study demonstrated pronounced loss of hair cells and spiral ganglion neurons in the base of the inner ear of aged ERβ-deficient mice compared to WT, accompanied by a greater hearing loss in the aged ERβ-deficient mice (Simonoska et al., 2009b). Recently, a report showed that 17β-estradiol protects the hair cells of rats against the ototoxic aminoglycoside gentamycin *in vitro*, and that this protection is mediated by ERs (Nakamagoe et al., 2011).

Complementary to the cellular and molecular effects, physiological evidence of estrogenic effects on the auditory system exists. An estrogen antagonist, tamoxifen, causes significant changes in contralateral suppression, as measured with distortion product otoacoustic emissions with increasing age in female mice (Thompson et al., 2006). Longer latencies in auditory brainstem responses (ABR) in ovariectomized rats compared to intact rats are reversed by estrogen treatment, and the subtle interpeak latency differences suggest hormonal involvement in a central as well as a peripheral level (Coleman et al., 1994). Moreover, studies of perceptual functions, which entail cognitive elements and their related behavioral outcome, report the effect of hormonal status on the perception of behaviorally relevant auditory signs and maternal behavior in mice (Koch and Ehret, 1989, Ehret and Schmid, 2009, Miranda and Liu, 2009).

1.4.2 Clinical evidence

Basic science analyses suggest predominantly protective effects of estrogens on hearing, and human studies agree for the most part. Menopause, which is a state of low estrogen levels, lasts for over three decades in many women's lives and is accompanied by a vulnerability to age- and hormone-related diseases. There is concern that hearing is also negatively affected by this hypoestrogenic state. At present, knowledge of the benefits and risks of hormone replacement therapy (HRT) for the hearing of postmenopausal women is insufficient, and several studies with their focus on HRT effects on hearing have been performed with controversial results. One of the earlier investigations is a prospective study examining the effects of either estrogen therapy or a combination of estrogen and progestin on the hearing sensitivity of postmenopausal women (Kilicdag et al., 2004). This study, relatively small in number of subjects, suggests that estrogen therapy delays age-related hearing loss in postmenopausal women. In a more recent retrospective study (Guimaraes et al., 2006) the hearing ability of postmenopausal women receiving combined hormone treatment (estrogen and progestin) was compared to that of an estrogen-only treated group and a control group. A battery of hearing tests included pure-tone audiometry, tympanometry, distortion-product otoacoustic emissions (DPOAEs), transient otoacoustic emissions, and the hearing-in-noise test (HINT). Pure-tone thresholds, DPOAEs and the HINT results show poorer performance for the combined estrogen and progestin group compared to the estrogen and the control groups, suggesting that the addition of progestin has a negative influence on both the peripheral and central auditory system, whereas estrogen has neither a protective nor a deleterious effect on hearing. Another study demonstrates a potential protective effect of HRT on hearing, but no information on the constituent hormones of the prescribed HRT is given (Hederstierna et al., 2007). Evaluation of the effects of estrous cycle and combined oral contraceptive pills on ABR, shows shorter latencies and inter-peak intervals in ABR waves during the periovular phase, compared to the luteal phase and in women under contraceptive treatment (Caruso et al., 2003b). Similarly, when women under surgically-induced

menopause are treated with estrogen therapy, ABR wave latencies are confirmed to be shorter (Caruso et al., 2003a). Taken together, epidemiological data from human studies indicate a reduced risk of hearing loss in women treated with estrogen or hormone therapy at the time of menopause or an improvement of hearing function in high estrogen conditions, even though evidence is contradictory at times.

Subtle changes in auditory perception during the menstrual cycle have long been suggested by Haggard and Gaston, who showed that low-frequency tones and interaural time differences for sound localization are particularly susceptible to the biochemical changes of the menstrual cycle (Haggard and Gaston, 1978). More recently another study suggested higher hearing sensitivity in the periovulatory phase, using a battery of hearing tests including otoacoustic emissions (OAE), medial olivocochlear (MOC) suppression and ABR (Al-Mana et al., 2010). An additional report examining ABR during the menstrual cycle, confirms shorter wave latencies and interpeak intervals in the periovulatory phase (Serra et al., 2003), and a study reports menstrual cycle-related changes in auditory event-related potentials, with most prominent changes occurring in the luteal phase (Walpurger et al., 2004).

Sex variation is also apparent in the auditory system. Sex differences in ABR are well-documented, with longer latencies and lower magnitudes for the males (Jerger and Hall, 1980). These differences were initially thought to stem from the smaller head circumference and shorter length of the brainstem pathway between the auditory nerve and midbrain in females compared to males (Jacobson, 1985). Evidence of a hormonal component of sex differences in ABR was later added to these morphological causes, from studies showing differences between younger and older women, as well as studies showing that postmenopausal women have longer auditory brainstem response latencies than age-matched men when compared to younger women or men (Jerger and Johnson, 1988, Wharton and Church, 1990). Additionally, when hearing sensitivity is evaluated by pure tone audiometry in postmenopausal women, an association is found between low serum estradiol or bone mineral density and increased risk of hearing loss (Kim et al., 2002, Walpurger et al., 2004). Some differences by sex and by sex-differentiation, that the auditory system manifests, are thought to derive from prenatal exposure to androgens (McFadden, 2011). For example females have more (and stronger) OAEs than males at birth as well as in adulthood (McFadden, 2009). It has been suggested that these differences derive from hormonal exposure *in utero*, as it is shown that prenatal exposure of females to high levels of androgens of their opposite sex twin, is correlated with masculinizing effects of their spontaneous acoustic emissions (McFadden, 1993).

Other studies focusing on hearing problems in patients with Turner's syndrome (a genetic condition in which a female lacks all or part of an X chromosome), suggest that the hypoestrogenic state that characterizes this syndrome may be partly responsible for the frequent hearing problems of these patients (Hultcrantz et al., 1994, Hultcrantz, 2003, Hederstierna et al., 2009). Additionally, Symptoms of Meniere's disease, which affects the inner ear and is characterized by hearing loss and balance disabilities, are exacerbated during the premenstrual phase, when estrogen levels are low (Andrews et al., 1992).

1.5 BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

BDNF is a neurotrophic peptide synthesized in neurons. It is required for the development, maintenance of plasticity and synaptic connectivity of the nervous system. Its functions are mediated by the Trk tyrosine kinase receptors and its synthesis is regulated by neuronal activity and hormonal influences (Lindholm et al., 1994, Thoenen, 1995, Huang and Reichardt, 2001). One mechanism through which estrogens

exert their neurotrophic effects was revealed by the discovery of an ERE in the gene encoding BDNF (Sohrabji et al., 1995). In the same time, it was found that mice lacking BDNF develop with degeneration of type 2 spiral ganglion neurons and loss of outer hair cell innervation (Ernfors et al., 1995). Construction of BDNF-mutant mice, which suffered from significant hearing loss, further shows the importance of BDNF throughout life for the maintenance of hearing neurons (Ernfors et al., 1994, Agerman et al., 2003). BDNF improves survival of auditory neurons *in vitro* (Lefebvre et al., 1994, Staecker et al., 1995) and demonstrates therapeutic potential against cisplatin toxicity in both auditory neurons and hair cells *in vitro* (Gabaizadeh et al., 1997). Survival of SGNs of guinea pigs exposed to the ototoxic combination of an aminoglycoside antibiotic and a loop diuretic, improves after intracochlear infusion of BDNF (Staecker et al., 1996). A similar model of chronic administration of BDNF via a mini-osmotic pump into scala tympani resulted in improved SGN survival (Miller et al., 1997). Geschwind and colleagues, efficiently expressed BDNF in many cell types, including auditory neurons, and demonstrated secretion of bioactive BDNF in primary murine SGN explants accompanied with neuritic process outgrowth, highlighting the possibility of BDNF as adjunctive gene therapy for deafness (Geschwind et al., 1996). The therapeutic potential of BDNF in the auditory system was further shown in a study, where BDNF gene therapy prevented degeneration of auditory neurons caused by intracochlear neomycin injections (Staecker et al., 1998). Even though the protective role of BDNF in auditory neurons has been repeatedly demonstrated, the potential link between estrogen and BDNF neuroprotection in the auditory system has not been further explored.

2 AIMS OF THE STUDY

The overall aim of this study was to determine whether estrogen receptors are present in the central and peripheral auditory system and to investigate their functional role.
Specific aims:

I. To localize estrogen receptors in the auditory structures of the central auditory system and establish possible sex differences in their expression patterns.

II. To evaluate the expression patterns of estrogen receptors in the central auditory system of different age populations characterized by low hormonal levels, namely prepubertal and aged mice.

III. To analyze the biochemical effects of ovariectomy and 17β-estradiol replacement therapy on the content of estrogen receptors in central and peripheral parts of the auditory system of mice, along with functional effects on auditory-related behavior.

IV. To explore the role of estrogen receptors and brain-derived neurotrophic factor (BDNF) in neuroprotection of the auditory system against acoustic trauma.

V. To explore the effect of fluctuating estrogen levels during the estrous cycle, on the expression of estrogen receptors in the auditory system.

3 METHODOLOGICAL CONSIDERATIONS

3.1 ANIMALS

In order to examine the expression of ERs in the central auditory system by immunohistochemistry, CBA/Ca mice of both sexes aged either 4 weeks or 6-9 weeks were supplied by Scanbur (papers I and II) and CBA aged 26-28 months were raised from birth within the University of Rochester Vivarium (paper II). CBA mouse strain is the golden standard for studying the neural, molecular and genetic bases of age-related hearing loss, because it retains its hearing ability until late in age like most humans. CBA female mice 10-14 weeks old were supplied by Charles River (Germany) for the molecular and behavioral analyses after ovariectomy and 17β-estradiol treatment (paper III). CBA female mice were supplied by Charles River (Germany) and Harlan (Netherlands) for the analyses of the expression of ERs in the auditory system during the estrous cycle (preliminary results). Wild type and homozygous mutant mice lacking the genes for ERβ (BERKO), ERα (ERKO), and aromatase (ARKO), between 12 and 22 weeks of age were used to examine the role of ERs and BDNF in neuroprotection of the auditory system against acoustic trauma (paper IV). BERKO and ERKO mice were supplied by Taconic. ARKO mice were bred at the animal facility at Huddinge hospital of the Karolinska Institutet. All experiments were approved by and conducted in accordance with the guidelines of the Stockholm Animal Research Ethical Committee or the University of Rochester's Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

3.2 EVALUATION OF HORMONAL PROFILES DURING THE ESTROUS CYCLE

Phases of the estrous cycle of mice were determined by vaginal lavage and microscopical examination of the presence of leukocytes, nucleated, and cornified cells in the obtained smears (Champlin et al., 1973, Caligioni, 2009) (Fig. 6). Proestrous is characterized by the presence of round nucleated cells and corresponds to the time before ovulation, when estradiol, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) increase. Estrous consists of cornified epithelial cells, which lack visible nucleus and have an irregular shape. Estradiol levels fall back to basal levels in this phase. Metestrous consists of all three types of cells, namely leukocytes, nucleated epithelial and cornified epithelial cells. Estradiol levels are still low. Finally, diestrous is characterized by leukocytes and by increasing estradiol levels towards the end of this phase. Direct microscopical examination of the

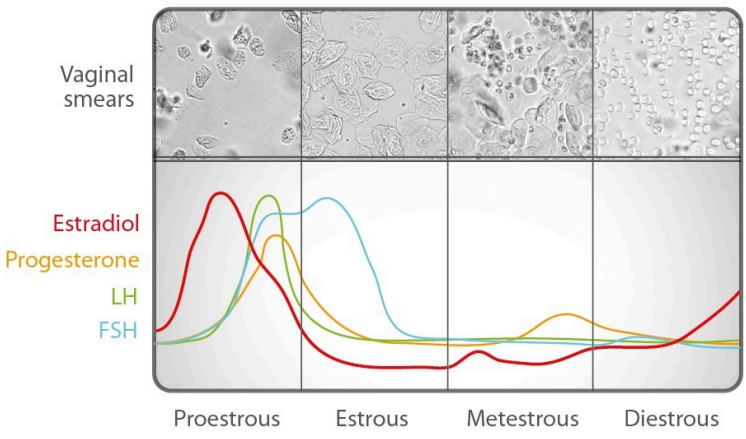

Figure 6. The 4-day estrous cycle of the mouse. Hormonal fluctuations are accompanied by changes in cytology of vaginal smears. Adapted from (Neill and Knobil, 2006).

obtained smears was preferred over costly and time-consuming Papanicolaou and methylene blue staining techniques, since it has been shown to yield the same results (Yener et al., 2007).

3.3 IMMUNOHISTOCHEMISTRY

The following primary antibodies were used: polyclonal rabbit anti-ERα antibody (MC-20 sc-542; Santa Cruz Biotechnology) raised against a peptide mapping at the C-terminus of ERα of mouse origin, polyclonal rabbit anti-ERβ antibody raised against residues C(467) to C(485) in the ligand-binding domain of rat ERβ (PA1-310B; Affinity Bioreagents), polyclonal rabbit anti–ERβ antibody (ab3577; Abcam), monoclonal mouse anti-ERβ antibody (ab288; Abcam), polyclonal rabbit anti-aromatase antibody against C-terminus of human aromatase (ab35604; Abcam). Secondary antibodies were followed by an immunoperoxidase reaction (VectaStain ABC kit PK-6100 and DAB substrate kit SK-4100; Vector Laboratories). The specificity of the antibodies used for these studies was verified by several control experiments. Substitution of the primary antibodies by blocking solution showed no immunoreactivity. Likewise, pre-incubation of anti-ERα and anti-ERβ antibodies with ERα-blocking peptide (sc-542 P; Santa Cruz Biotechnology), ERβ-blocking peptide (PEP-007; Affinity Bioreagents) or ERβ-blocking peptide (catalog no. ab3564; Abcam) respectively, showed no immunoreactivity in both immunohistochemistry and western blot providing further evidence for the specificity of the antibodies. Immunolabeling of tissue from ovaries, where the expression pattern of each antibody is distinct and well established, was used to further validate antibody specificity, using standard immunocytochemical methods. Cochlear sections from BERKO mice were used as a negative control for anti-ERβ antibody. The effects of different fixatives on the staining pattern of in the cochlea were also tested. These different fixatives (4% paraformaldehyde/1% acetic acid or 4% paraformaldehyde/1% acetic acid/0.1% glutaraldehyde) in PBS were compared with our standard fixation with 4% paraformaldehyde in PBS. Specificity of anti-ERα and anti-ERβ antibodies was further confirmed by western blot with ovaries as positive controls. All efforts were made in order to keep consistency in the processing of the prepubertal and aged brains.

The stereotaxic map of the mouse brain (Franklin and Paxinos, 2008) was used as a reference in order to accurately define the different structures throughout the central auditory pathway. In accordance with previous studies of ERs distribution in the CNS (Mitra et al., 2003), an estimate of the distribution and relative density of immunoreactive cells (++++ very abundant, +++ abundant, ++ present, + few, - not present), as well as of the intensity of signal obtained (++++ intense, +++ high, ++ medium, + low immunoreactivity) was made. In order to control for the influence of methodological variation on the intensity of immunostaining, we assessed regions where strong immunoreactivity of each ER is well established (thus characterized as very intense or ++++). These regions include: (a) the periaqueductal gray matter in the midbrain for ERα (only a few ERβ-positive neurons), (b) the Purkinje cells of the cerebellar cortex in the hindbrain for ERβ (negative for ERα), and (c) the medial amygdala in the forebrain for both ERα and ERβ.

3.4 WESTERN BLOT

To obtain a total protein extract, cochleae from 2-5 mice were pooled. Protein fractions containing 19–28μg total protein per sample were electrophoresed under reducing conditions. The primary antibodies used for western blot were polyclonal rabbit anti-ERα antibody (MC-20 sc-542; Santa Cruz Biotechnology), polyclonal rabbit anti-ERβ antibody (PA1-310B; Affinity Bioreagents) and polyclonal rabbit anti-ERβ

(developed at Department of BioSciences and Nutrition, Novum, Karolinska Institutet, the specificity of which was verified by anti-ERβ 530 from Panvera as a positive control and tissue from BERKO cochlea as a negative control). Mouse monoclonal anti-GAPDH IgG (ab9484; Abcam) and mouse monoclonal anti-TBP IgG (catalog no. ab818; Abcam Ltd.) were used as loading controls of the cytosolic and nuclear fractions respectively. Secondary antibodies included horseradish peroxidase (HRP)-conjugated antibodies or a mixture of IRDye800-conjugated anti-rabbit IgG and IRDye680-conjugated anti-mouse IgG, depending on the detection system used. Detection systems included: a) an enhanced chemiluminescence western blot detection kit (catalog no. 34076; SuperSignal West Dura; Pierce Biotechnology) visualized with either Lumi-Film chemiluminescent detection films (Roche Diagnostics) or a chemiluminescence detection camera (GelDoc; BioRad), and b) direct infrared fluorescence detection at 700 and 800nm channels (Odyssey Infrared Imaging System, LI-COR Biosciences, Lincoln, NE, USA). The latter allows for simultaneous detection of different proteins. GenTools (SynGene, Cambridge, England) was used for the quantification of the bands. Data was statistically analyzed with ANOVAs and post hoc comparisons were carried out with Tukey-Kramer HSD test and statistical significance level was set at $p<0.05$.

3.5 ANESTHESIA AND SURGICAL PROCEDURES

To explore the molecular and behavioral effects of circulating estrogens in the auditory system, adult (10 weeks old) female mice were bilaterally ovariectomized or sham operated. Mice were administered an intraperitoneal (i.p.) injection of ketamine (Ketalar, 100 mg/kg) and xylazine (Rompun, 8 mg/kg) and left in a calm environment for 10-15 minutes for induction of anesthesia. The animal's reaction to hind paw pinching and its breathing pattern were used to determine the level of anesthesia. The mid-dorsal area was shaved and the skin was disinfected with ethanol. A midline dorsal skin incision at the lower 1/3 of the back was followed by lateral muscle incisions on both sides of the body. Both ovaries were removed and the uterine horns were returned into the peritoneal cavity. Muscle wall and skin were sutured with resorbable suture material. For the sham surgery the same procedure was followed except for the fact that ovaries were exposed but not removed. In those cases when not all mice of a single cage were operated in the same day, mice were single-caged after surgery to fully recover from anesthesia and surgery before they rejoined their cages. Post-operative analgesia consisted of buprenorphine (Temgesic, 0.05mg/kg) administered s.c. for three days.

Body weight was measured at the day of surgery (10 weeks old) and at the day of sacrifice (13 weeks old). The undeniable effects of circulating estrogens on metabolism and body fat are well known (Wade and Schneider, 1992) and this is the reason why weight gain is commonly used as a confirmatory measure of ovariectomy and estrogen replacement therapy. The relevance of body weight in ASR measurements of the present study is also of interest. Despite significant body weight differences between groups, ASR data was not normalized to weight. Differences in musculature rather than fat are of relevance for the study of startle responses, whereas weight gain in the OVX mice is a result of increased body fat rather than muscle mass. Thus, we reckon that it is unlikely that body weight differences induced by OVX and hormone replacement would influence startle responses. Musculature differences caused by sex and age differences are avoided in our study by the single use of females of the same age.

3.6 PHARMACOLOGICAL TREATMENTS

Pellets containing synthetic 17β-estradiol (0.25 mg, 21 days; cat. No. E-121, Innovative Research of America) or placebo for 17β-estradiol [(0.25) mg, 21 days; cat. No. C-111, Innovative Research of America], were implanted s.c. in the lateral side of the neck immediately after the surgical procedures. 21 days are considered an adequate treatment duration that generates effects similar to chronic exposure to estrogens. The dosage was selected based on information that it yields levels of estrogens within the physiological range, supplied by the manufacturing company. The selective agonist for ERα propyl (1H) pyrazole-1,3,5-triyl-trisphenol (PPT) (catalog no. 1426, TOCRIS Cookson), and the selective ERβ agonist 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN) (catalog no. 1494, TOCRIS Cookson) were dissolved in DMSO (1%) and injected daily at a dose of 1 mg/kg for 1 week, whereas control animals for this experiment were treated with DMSO alone.

3.7 BEHAVIORAL TESTS

Acoustic startle response (ASR), Prepulse Inhibition of the startle response (PPI) and Startle Habituation were determined on the third week after the operation, which is also the beginning of hormonal treatment. The same person conducted all experiments in order to diminish handling stress of the animals and reduce variation in the results.

Apparatus

The acoustic startle apparatus consisted of two startle chambers (SR-LAB; San Diego Instruments, San Diego, CA), each containing a cylinder mounted on a frame, wherein the animal was placed. The cylinder allowed the animal to turn around freely without constraint. A loudspeaker mounted on the ceiling of the chamber delivered broadband background noise and acoustic stimuli. A piezoelectric accelerometer attached below the frame, detected vibrations caused by movement of the animal.

Protocol

All groups were balanced across the two startle-chambers. Startle testing took place during the light period, between 11:00 and 14:00. Animals were pre-exposed to a short pretest session in order to familiarize with the testing procedure one day before the actual testing. The pretest session consisted of a 5 min acclimatization period followed by 24 trials (15 “pulse-alone” and 5 “prepulse pulse” trials with a prepulse intensity of 12 dB above background noise). A schematic diagram of the experimental session is seen in figure 7. The session began with a 5 min acclimatization period to 65 dB background noise, which was continued throughout the session. The animal was then presented with a series of trials in pseudo-random order at variable intervals (7 - 23 sec, average 15 sec) lasting approximately 15 minutes. Five different types of trials were presented: “pulse-alone” trials, in which a 120 dB broadband burst of 40 msec duration was presented; three types of “prepulse pulse” trials, in which a 20 msec broad-band prepulse 3, 6, or 12 dB above background noise preceded the onset of the 120 dB pulse by 100 msec; and a “no stimulus” trial, which included only the background noise. The test session was divided into four blocks: the first block consisted of 5 “pulse-alone” trials in the beginning of the session, the second and third blocks each included 6 “pulse-alone” trials, 15 “prepulse pulse” trials (5 of each type) and 4 “no stimulus” trials, and the fourth block consisted of 5 “pulse-alone” trials at the end of the session.

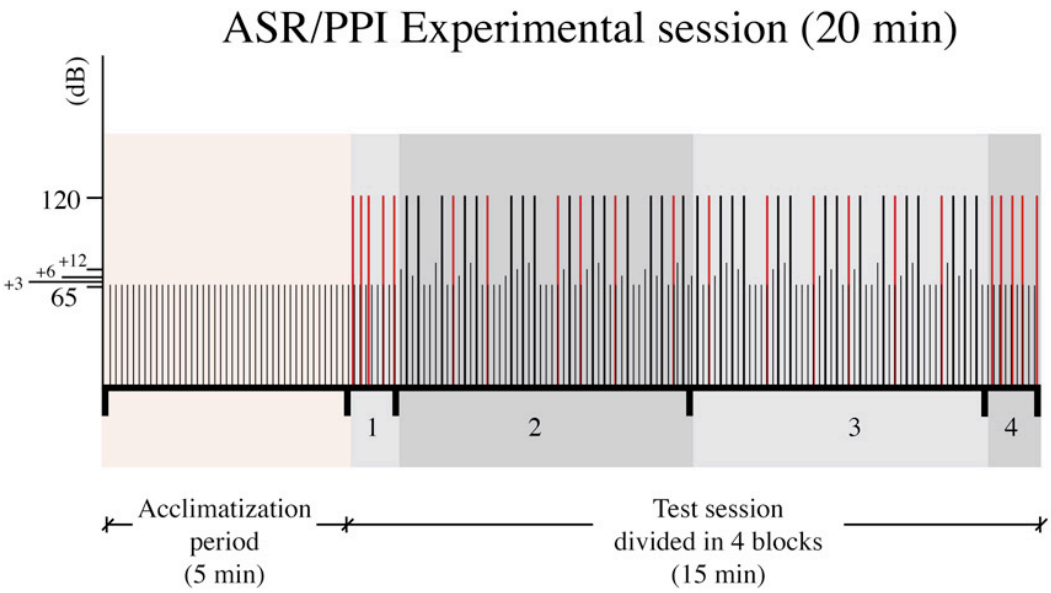

Figure 7. Schematic representation of acoustic startle response (ASR) experimental protocol. Red lines extending to 120dB represent "pulse-alone" trials, black represent "prepulse pulse" trials.

Data Analysis

For each "pulse-alone" and "prepulse pulse" trial, the startle response to the 120 dB burst was recorded. The following measures were then calculated from these data for each animal. Startle magnitude refers to the mean maximal startle response (V_{max}) to "pulse-alone" trials within the middle two blocks, measured in volts (V). Startle latency (T_{max}) represents the mean latency to the maximal response to the "pulse-alone" trials within the middle two blocks, measured in milliseconds (msec). PPI refers to the decrease in the startle response with the presentation of a prepulse stimulus, which is presented shortly before the pulse stimulus and is typically too weak to elicit a startle response itself. PPI was calculated for each type of "prepulse pulse" trial as a response for "prepulse pulse" trial) / mean maximal startle response for "pulse-alone" trial] x 100. Habituation effects were avoided in calculation of ASR and PPI by including data from the middle portion of the session and when the level of startle reactivity is most stable and representative and no habituation effects are present (Geyer et al., 1990). Habituation of the startle reflex was assessed as the percentage of decrease in startle response throughout the session by comparing the mean maximal startle amplitude of five "pulse-alone" trials presented at the first and last block of the startle session, using the equation: Habituation = 100 – [(mean maximal startle response in block 4 / mean maximal startle response in block 1) x 100]. Data was analyzed with analyses of variance (ANOVAs) and post hoc comparisons of means were carried out with Tukey-Kramer HSD test. Statistical comparisons were carried out at a significance level of $p < 0.05$.

3.8 QUANTITATIVE RT-PCR

To quantify expression of ESR1 and ESR2 genes, cochleae were dissected in RNAlater (Qiagen) under the microscope. The outer bony shell was removed together with the stria vascularis. The eventual cochlear sample consisted of the modiolus containing the spiral ganglion, parts of the basilar membrane and the organ of Corti. Two to ten cochleae were pooled per sample in order to get adequate RNA

concentrations. Total RNA was extracted immediately after the dissections using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Spectrophotometry [NanoDrop 1000 Spectrophotometer (Thermo Scientific)] was used to determine RNA concentrations and RNA quality. Samples were excluded from the study either due to lower purity [(A260/280 ratio < 1.8; (absorbance at 260/280 nm)] or low RNA concentration, which was inadequate for cDNA synthesis. Cochlear nuclei and inferior colliculi were stored at -80^{0}C until RNA extraction with Trizol reagent (Invitrogen). RNA was reverse-transcribed using Superscript III (Invitrogen). Complementary DNA (cDNA) was generated from total RNA using random hexamers and SuperScript III (Invitrogen). Quantitative real-time reverse transcription PCR was conducted on an Applied Biosystems 7500Fast instrument in the 'Relative quantification (ddCt) plate' mode using TaqMan Universal PCR Master Mix (Applied Biosystems) under standard conditions. Pre-developed specific assays (Taqman gene expression assays, Applied Biosystems) labeled with FAM as a reporter dye were used to determine expression of ESR1 (Mm00433149_m1), ESR2 (Mm00599821_m1) and Brain-derived neurotrophic factor (BDNF) (Mm01334042_m1). For each cDNA sample, three internal controls were also measured in the same plate to ensure comparable cDNA amounts in all wells: Hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (Mm00446968_m1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) and beta actin (Mm00607939_s1). All reactions were performed in triplicates. Both negative reactions (templates without reverse transcription enzyme) as well as no-template-controls (including each primer and master mix but no template) were performed on every plate to check for contamination and showed no amplification. Relative expression of target genes compared with the most stable of the three internal controls in each sample was calculated (ΔCt) and fold-changes of target genes among samples were determined by calculating the difference in ΔCt between samples (ΔΔCt) (Schmittgen and Livak, 2008) and statistically analyzed with ANOVAs. Post hoc comparisons were carried out with Tukey-Kramer HSD test and statistical significance level was set at $p < 0.05$.

3.9 ACOUSTIC TRAUMA

The acoustic trauma used in this study caused a temporary change in auditory threshold shifts. This type of trauma does not lead to any morphological changes such as hair cell loss or degeneration of spiral ganglion neurons, and complete recovery of ABR thresholds occurs by 48 h. We should note though that recent advances reveal that progressive consequences of 'temporary' noise-induced hearing loss are considerably broader than what was previously thought and may be masked by normal hearing threshold sensitivity (Kujawa and Liberman, 2009).

4 RESULTS

4.1 ERS IN THE CENTRAL AUDITORY SYSTEM OF MICE (PAPER I)

An anatomic map of ERα and ERβ expression in the central auditory system was constructed (Fig. 8). Expression of ERα and ERβ was shown in most regions directly involved in auditory processing in the central nervous system. Young adult mice of both sexes were tested to determine whether differences between sexes exist. Similar patterns of ERs were found in both males and females and both ERs were extensively expressed throughout the central auditory system.

ERα immunoreactivity was observed in a large number of areas related to auditory processing. Strongly immunopositive neurons were found in auditory nerve nucleus (AuN). The posterior part of ventral cochlear nucleus (VCP), the nucleus of the trapezoid body (Tz), lateroventral periolivary nucleus (LVPO) and medioventral periolivary nucleus (MVPO) were also positive for ERα. The dorsal nucleus of the lateral lemniscus (DLL) showed strong cell nuclear staining. In the inferior colliculus (IC), the central nucleus (CIC) was negative, the dorsal cortex (DCIC) and anterior part of the external cortex (ECIC) had a few strongly positive cells, and the brachium (BIC) showed pronounced ERα immunoreactivity. The medial geniculate nucleus (MGN) of thalamus was negative for ERα and the auditory cortex (AuC) contained a few large pyramidal neurons, together with smaller cells in the outer layers of cortex.

ERβ immunoreactivity was present throughout the central auditory pathway. The neurons of the AuN close to the posterior part of the VCN exhibited the strongest immunoreactivity for ERβ. VCP showed low to moderate immunoreactivity, whereas VCA, DCN, and the granule cell layer were

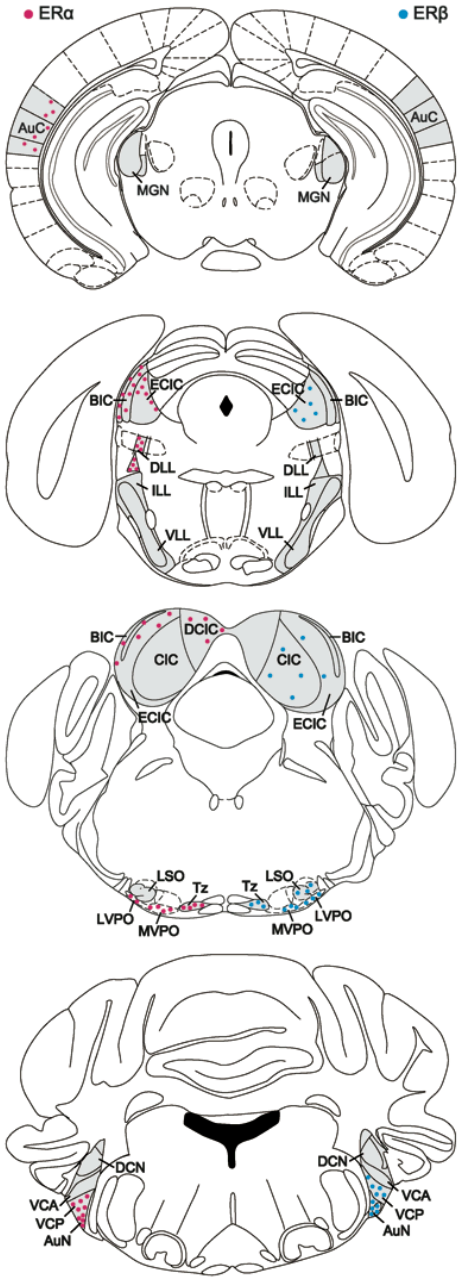

Figure 8. Schematic summary of the presence of ERα and ERβ in the central auditory system. Pink dots represent ERα and blue dots ERβ immunoreactivity. The diagrams of coronal brain sections are modified form the mouse brain atlas of (Franklin and Paxinos, 2008)

negative. Moderate immunoreactivity was observed both in the nucleus and cytoplasm of the neurons of Tz. A gradient was observed, with a larger number of darkly stained cells in the lateral part of the Tz, which is related to the processing of low frequencies, compared to the medial part of the Tz, which processes high frequencies. LSO showed moderate immunoreactivity for ERβ. MSO and SPO were negative for ERβ, whereas the rest of the SOC complex, including LVPO and MVPO, were positive. DLL and VLL stained weakly for ERβ. Less immunoreactivity for ERβ compared to ERα was observed in the inferior colliculus. The central nucleus and external cortex of the IC contained most of the positive cells, whereas the brachium and the dorsal cortex of the IC were negative. MGN was immunonegative for ERβ, as was the auditory cortex.

Cytoplasmic staining of aromatase was found throughout the neurons in the central auditory pathway without obvious differences between males and females. Strong aromatase staining was found in the AuN. VCP and VCA showed moderate immunoreactivity. The Tz showed intense immunoreactivity and LVPO showed moderate staining. LL was strongly positive in both the dorsal and ventral nucleus. All regions of the IC, including the central nucleus, dorsal cortex, external cortex and the brachium were moderate to strongly immunoreactive. MGN was negative and the auditory cortex had a few large pyramidal neurons positive for aromatase.

4.2 ERS EXPRESSION PATTERNS CHANGE WITH AGE (PAPER II)

Immunolocalization of ERs was expanded in prepubertal and aged CBA mice. The localization patterns differed between two groups, and differences were also found when compared to young adults. No differences were observed between male and female mice at any age group.

In the prepubertal mice the lower parts of the central auditory system were mostly negative for ERα. CN was largely negative for ERα with only the granule cells in its dorsal part showing ERα immunoreactivity. The subnuclei of SOC were also negative. DLL was strongly positive, as were most parts of IC, namely the DCIC, BIC and ECIC, whereas CIC was negative. MGN was negative and AuC contained ERα positive neurons. On the other hand ERβ was mostly found in the lower parts of the central auditory pathway. AuN, VCP and VCA showed some ERβ immunoreactivity. Subnuclei of the SOC and lateral lemniscus showed low to moderate ERβ immunoreactivity. Higher structures including subdivisions of IC, MGN and the auditory cortex were negative for ERβ.

In the aged group, DCN and VCA were negative for ERα. AuN and VCP showed immunoreactivity of medium and low intensity respectively. ERα immunoreactivity was observed in the Tz, SPO, MVPO and LVPO, while LSO and MSO were negative. DLL was positive whereas the ventral and inferior nuclei were negative. In the IC the CIC was negative whereas some immunoreactivity was seen in the commissure of the DCIC. ECIC and BIC showed medium and strong immunoreactivity, respectively. MGN and the AuC were negative. Regarding ERβ in the aged mice it was found in the VCP and VCA. The AuN showed strong ERβ immunoreactivity. All parts of the SOC were positive with the Tz and the periolivary nuclei showing slightly higher expression than the other regions. DLL and VLL had some immunopositive cells of low intensity. Most divisions of IC (CIC, ECIC and BIC) showed low intensity immunoreactivity, while DCIC was negative. The MGN and the AuC were devoid of ERβ.

4.3 MOLECULAR AND BEHAVIORAL EFFECTS OF 17β-ESTRADIOL TREATMENT (PAPER III)

Endocrine ablation (ovariectomy) and hormone replacement therapy (17β-estradiol treatment) were employed to test the hypothesis that circulating sex hormones

affect ER levels in the auditory system and have a potential role in auditory function. ER levels were measured in RNA extracts of dissected cochleae and inferior colliculi with qRTPCR. Chronic 17β-estradiol treatment caused a significant decrease in ERα but not ERβ RNA levels in the cochlea (see figure 1 of paper III) and the inferior colliculus (see figure 2 of paper III) of ovariectomized mice compared to placebo treatment. No significant differences were noted between sham-operated and ovariectomized mice. The lack of such an anticipated result may be partly explained by the high diversity of hormonal profiles of the sham-operated group, since these animals were tested randomly as far as the phase of their estrous cycle is concerned.

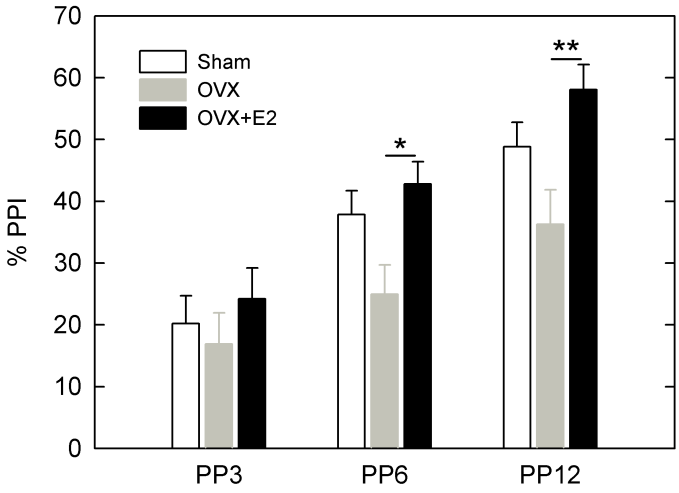

Figure 9. Mean ± SEM % PPI at different prepulse intensity levels in sham-operated, ovariectomized (OVX) and OVX+E2 mice (n = 38). Data are means ± SEM. * $p < 0.05$ and ** $p < 0.01$ by ANOVA.

Furthermore, we sought functional evidence of the molecular changes induced by OVX and 17β-estradiol treatment in the auditory system, and therefore assessed a number of behavioral parameters related to acoustic stimulation. Chronic 17β-estradiol replacement therapy caused differences in auditory-related behavioral parameters compared to placebo treatment in ovariectomized mice. The amplitude of acoustic startle response was not influenced by 17β-estradiol treatment (see figure 3 of paper III), whereas the latency of ASR increased in the 17β-estradiol-primed compared to the non-primed (placebo) OVX mice (see figure 4 of paper III). Prepulse inhibition of the acoustic startle response increased in the 17β-estradiol-primed compared to the non-primed (placebo) OVX mice (Fig. 9). Again no reverse change was shown in the parameters in which differences were observed between the Sham and the OVX group. Nevertheless, a trend of decreased PPI in the OVX group when compared to intact animals (both treated with placebo) was present, even though not statistically significant. Startle response amplitude tended to decrease during the session in all groups. However only in the 17β-estradiol treated OVX mice was this decrease significant (see table 1 of paper III). When we compared the effect of treatment on habituation of startle response, it lacked statistical significance.

4.4 ER-MEDIATED PROTECTION FROM ACOUSTIC TRAUMA THROUGH BDNF (PAPER IV)

The role of ERs and BDNF in the protection of the auditory system against acoustic trauma was investigated. ABR thresholds were measured before and 24 hours after acoustic trauma on wild-type and multiple knockout mice. WT and homozygous mutant mice lacking the genes for ERβ (BERKO), ERα (ERKO), and aromatase (ARKO) were used. BERKO and ARKO mice showed significantly higher ABR threshold shifts after acoustic trauma than WT mice, whereas ARKO mice did not differ from WT mice, suggesting that lack of ERα does not affect the auditory system's response to acoustic trauma (Fig. 10). The role of ERs in hearing was further explored

by testing the potential of specific ER agonists in protection against acoustic trauma. ABR thresholds were measured before and 24 hours after acoustic trauma in ARKO and WT mice. DPN provided partial protection from acoustic trauma in WT mice (see figure 2 of paper IV). Both DPN and PPT treatment were protective in ARKO mice, albeit DPN to a larger extent. In order to reveal possible sites of ERβ action in the cochlea immunohistochemistry was performed and ERβ was localized in the spiral ganglion neurons, inner and outer hair cells.

Protective effects of estrogens in the nervous system are partly enacted via induction of neuroprotective pathways, such as the BDNF pathway. We hypothesized that the protective effects of estrogens in the auditory system may be a result of BDNF activation in the auditory system. BDNF protein expression was assessed in the cochlea and a trauma-induced decrease in both WT and BERKO was found (see figure 5 of paper IV). In order to further explore the involvement of BDNF in protection of hearing from acoustic trauma, WT, ARKO and ERKO mice were treated with DPN, and BDNF expression in the cochlea was evaluated. A DPN-induced increase in BDNF protein levels was observed in ARKO and ERKO mice, in parallel with a DPN-induced increase in BDNF mRNA levels in WT mice (see figure 6 of paper IV). These findings suggest an estrogen-induced BDNF neuroprotective mechanism against acoustic trauma. Previous studies have demonstrated protective effects of BDNF in the auditory neurons and the hair cells, but an estrogen-induced mechanism of BDNF action in the auditory system has not been previously shown.

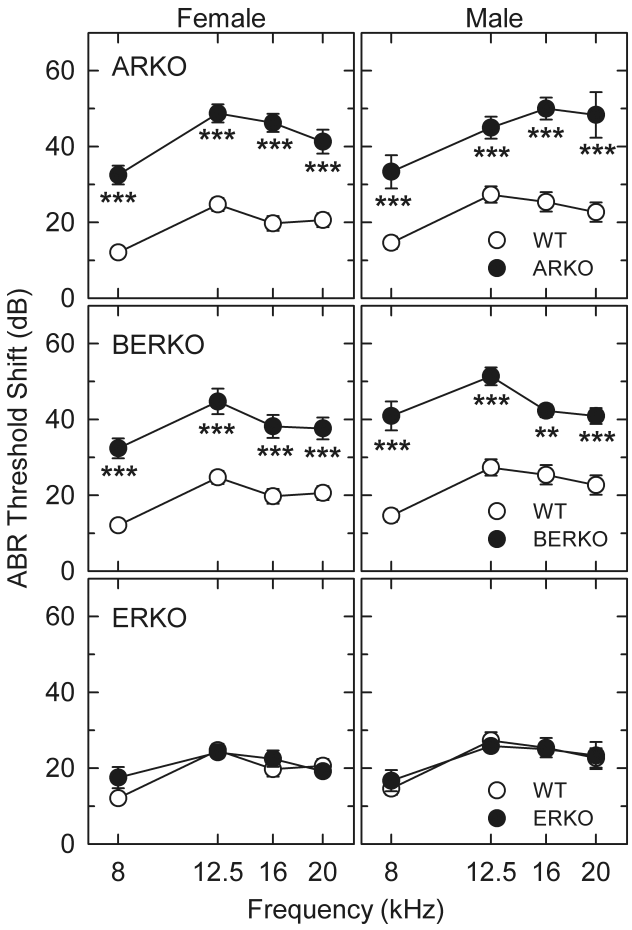

Figure 10. ABR threshold shifts 24 h after acoustic trauma. No differences were observed between males and females. Both ARKO and BERKO mice showed elevated threshold shifts at all frequencies compared to WT. ERKO mice had no differences in threshold shifts compard to WT. Data are means ± SEM. ** $p < 0.01$ and *** $p < 0.001$ by ANOVA.

4.5 BIOCHEMICAL CHANGES IN THE AUDITORY SYSTEM DURING THE ESTROUS CYCLE (PRELIMINARY RESULTS)

To further explore the potential impact of sex steroids on ERs and BDNF in the auditory system, we hypothesized that ER levels in the auditory system are modified by the ever-changing hormonal environment due to estrogen cyclicity. ERα and ERβ RNA levels in the peripheral auditory system of young CBA mice were examined during the

estrous cycle. Cochleae were harvested in the different phases of estrous cycle (proestrous, estrous, metestrous, diestrous) and qRTPCR analysis of ESR1 and ESR2 RNA was performed. Fine fluctuations in transcription of ESR1 mirroring the fluctuation of circulating estrogens were revealed (Fig. 11). ESR1 mRNA expression was significantly down-regulated in proestrous, when circulating estrogen levels peak compared to estrous and metestrous, which are characterized by low estrogen levels. ESR2 mRNA levels were stable and not affected by the hormonal changes of the estrous cycle. Furthermore the expression of BDNF was stable throughout the estrous cycle, similar to ESR2.

To further confirm these results, we evaluated ERα and ERβ protein expression in the cochleae of CBA mice during the estrous cycle using western blot. ERα protein expression was lower in proestrous, and there was an up-regulation of its levels towards metestrous (2.5-fold) (Figure 11), similar to the pattern of ESR1 mRNA fluctuation during the estrous cycle. Changes in protein expression of ERα were of a higher magnitude than ERβ.

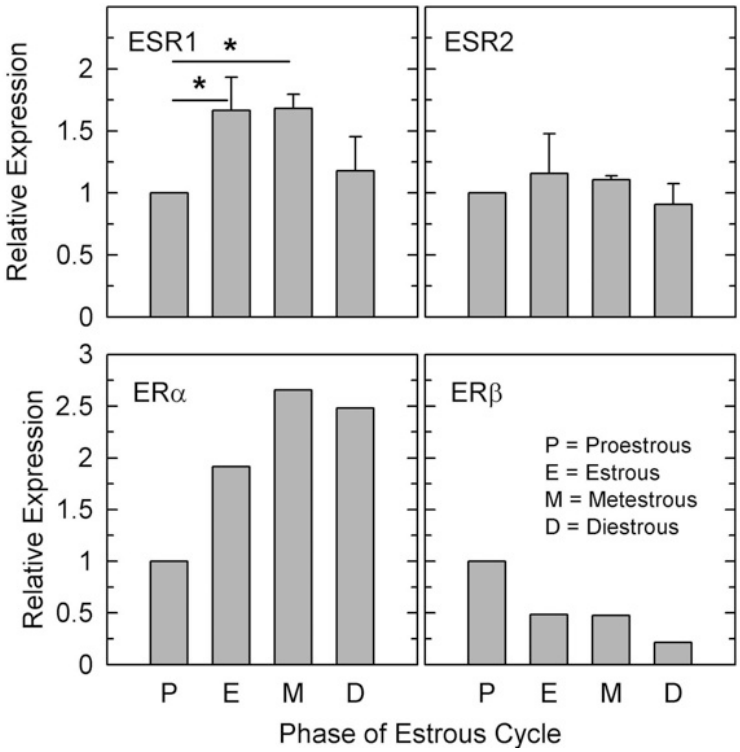

Figure 11. Relative expression of mRNA (ESR1 and ESR2) and proteins (ERα and ERβ) of ERs in cochleae during the estrous cycle: proestrous (P) (n=3), estrous (E) (n=3), metestrous (M) (n=2) and diestrous (D) (n=3). Error bars represent STD. * $p < 0.05$ by ANOVA. Each sample contains 6-10 pooled cochleae from 3-5 mice.

5 DISCUSSION

The results in this thesis demonstrate that the auditory system of mice is responsive to estrogens through estrogen receptors, which are present throughout the central and peripheral auditory system. Their expression patterns change throughout the life span, and these changes are not uniform but rather region-specific. The levels of circulating estrogens affect the expression of ERα in the peripheral and central auditory system and the behavioral outcome related to hearing. ERβ is not directly regulated by circulating estrogens, it is protective against acoustic trauma and this protection involves BDNF. Thus, estrogens affect multiple structures of auditory processing throughout life by fine-tuning basal auditory functions through alpha receptors and by protecting against acoustic trauma through beta receptors.

5.1 ESTROGEN RECEPTORS IN THE CENTRAL AUDITORY SYSTEM

Widespread expression of ERs throughout the central and peripheral auditory system indicates its high responsiveness to estrogens. In addition to estrogen-responsive neurons, estrogen-producing neurons (aromatase-immunopostive) are also abundant in the auditory system. The functional implications of such a widespread expression of ERs in the auditory component of the nervous system are largely unknown and require further studies. Estrogens have generally been correlated with neurotrophic and neuroprotective effects on the nervous system, and therefore the presence of estrogen receptors in the auditory pathway is likely to be associated with such protective effects. Since the specific knockout mice for each receptor tested in paper IV retained normal hearing, the presence of ERs throughout the central auditory system is more likely related to a fine-tuning role under normal conditions or a protective role when the auditory system is challenged.

No sexual dimorphism in the expression of ERs throughout the central auditory system was shown. In order to decrease variability caused by fluctuating estrogen levels, young females of reproductive age were sacrificed at a particular stage of their estrous cycle. Proestrous was chosen, because circulating estrogens are at their peak in this phase. The rationale was that sex differences if existent in the central auditory nuclei would then become more apparent, as the contrast in hormonal profiles between sexes would be maximized. The lack of systematic sex differences in the distribution of ERs in the auditory system presented here is not surprising and has been reported in previous studies in the CNS of rodents. Male and female gonadectomized rats have equivalent ER binding capacity in the hypothalamus, and sex differences appeared only in response to estrogen treatment (Brown et al., 1988, Yuan et al., 1995). No sex differences in the intensity of ER immunoreactivity were observed in the hippocampus of gonadectomized rats (Weiland et al., 1997) or in the brainstem or spinal cord of mice (Vanderhorst et al., 2005).

Even though sex differences in the expression of ERs were absent in the central auditory pathway, sex differences in auditory perception, sound localization, auditory nerve latencies, and otoacoustic emission amplitudes have been repeatedly recognized (Haggard and Gaston, 1978, McFadden and Pasanen, 1998, Caruso et al., 2003b, Thompson et al., 2006). One possible explanation for reported sex differences in the auditory function is sexual differentiation during early stages of development. Sex differences may either be a result of the hormonal differences at the time of testing, or a consequence of innate permanent sex differences during development. In the perinatal period, exposure to different levels of sex hormones permanently influences brain morphology and gender-typical behavior later in life. Phoenix et al were the first to

show 50 years ago, that masculinization (the acquisition of male-typical patterns) and defeminization (the loss of female-typical characteristics) of the brain happens early in life in response to sex steroid hormones in guinea pigs (Phoenix et al., 1959). In situ produced estrogens were demonstrated *in vitro* to play an important role in imprinting and neural differentiation in the mouse brain of both sexes (Toran-Allerand, 1980, Bakker et al., 2006), and more recent experiments in alpha-fetoprotein knock-out mouse showed that prenatal estrogen masculinize and defeminize the brain (Bakker et al., 2006). ERβ was found to be involved in defeminization of mouse brain and behavior (Kudwa et al., 2005). Therefore, even though no obvious sex dimorphisms in morphology of the auditory structures are apparent, it is possible that sex differences in the auditory function originate in early developmental processes.

In the light of the estrous cycle study, which shows a down-regulation of ERα in the cochlea during proestrous, it would also be interesting to study females in a phase of the estrous cycle other than proestrous. It could be that ER expression in females differs from males in some, but not all, phases of the reproductive cycle. Sex differences could be missed in male–female comparisons if the females are tested randomly across the reproductive cycle. Becker et al., extensively discussed these issues and suggested guidelines for studies on sex differences in brain and behavior (Becker et al., 2005). A five- instead of two-group design, and therefore a larger number of animals would be required to investigate this matter. This was not justified in our study, since our primary aim was to describe the existence of ERs in the central auditory system rather than detect subtle sex differences, for which a more quantitative method should be used.

5.2 MATURATION AND AGING

The expression pattern of ERs in the central auditory system of mice was assessed at two different age groups characterized by low circulating estrogens, namely prepubertal and aged mice. In the former group estrogen production has not yet been activated, whereas in the latter group, estrogen and testosterone levels are low because of multiple reasons: reduced production of hormones from the gonads because of diminished number of hormone-producing cells, decreased free hormone in the bloodstream because of increased concentration of the hormone-binding globulins and dysfunction of the hypothalamic-pituitary-gonadal axis (HPG) with blunted feedback response to low hormone levels (Vermeulen and Kaufman, 1995).

A clear dichotomy was observed in the expression of ERα and ERβ of prepubertal mice, with ERα mostly found in structures related to higher parts of auditory processing, and the opposite being true for ERβ. This phenomenon was not observed in the aged animals or the group of reproductive age (of paper I). The functional role of these distinct patterns for the two types of ERs may be related to maturation of the auditory system in this particular age, since it blunted after the onset of puberty. When comparing the distribution of ERs in the various structures comprising the central auditory system between the examined age groups, there is a lack of uniformity in the degree and direction of changes. This suggests that region-specific mechanisms rather than the overall hormonal milieu are responsible for these changes. Similar results have been previously demonstrated in other brain regions. Variations in hormone levels during the estrous cycle or in relation to sex, affected ER mRNA levels in neurons of the rat brain in a region-specific manner (Shughrue et al., 1992). Region-specificity of the ERs expression levels in different hormonal conditions, is a sign of the complexity of ER regulation in the brain, which cannot be explained simply by the endocrine components of the hypothalamic-pituitary-gonadal (HPG) axis, but includes further modulation at an autocrine or paracrine level.

Therefore, fluctuation of the hormone levels during the reproductive cycle, sex differences, systemic hormone replacement therapy or stages of maturation, are potential sources of variation of ER levels, still they compose only one part of the picture, the other part being the regional regulation of neurosteroids dependent on the needs of each brain region (Joels, 1997). The lack of sex differences in these two groups supports findings from paper I, in which no sex dimorphism was noted in mice of reproductive age.

The expression of ERs in the central auditory regions of the aged animals may be related to neurotrophic and neuroprotective effects on the remaining neurons. ERs in the aged brain are potential targets of neuroprotection of the brain. Menopause occurs as a gradual decrease in the levels of sex hormones, leading to irregular and progressively rare estrous cycles. In males there is also a substantial decrease in the levels of testosterone and estradiol with aging (Wu et al., 2009). The auditory function of the aged population is compromised, and decades of basic scientific research as well as dozens of clinical trials have demonstrated the benefits of estrogen action in the brain. Typically these benefits are elicited when estrogens are used as a preventive intervention on a healthy cell or organism, and not on an already compromised neurodegenerated foundation, as suggested by the 'healthy cell bias of estrogen action' hypothesis (Brinton, 2008). In the latter case, evidence from hormone intervention on women with deteriorated neurological function gave contradictory clues. The Women's Health Initiative Memory Study (WHIMS) suggested that combined HRT (estrogen plus progestin) increases risk for dementia, and drew attention on the effects of estrogen-alone HRT versus combined (estrogen plus progestin) HRT (Shumaker et al., 2003). A following study examined the effects of combined versus estrogen-only HRT on the hearing of postmenopausal women, and showed a negative effect of progestin, whereas estrogen had neither a negative nor a protective effect (Guimaraes et al., 2006). Thus, both the starting foundation and the actual compounds of hormone treatment need to be taken into consideration, when analyzing results from hormonal interventions in aged women.

5.3 MOLECULAR AND BEHAVIORAL EFFECTS OF ESTRADIOL ON THE AUDITORY SYSTEM

Molecular correlates of 17β-estradiol actions were assessed in cochleae and inferior colliculi. Estrogen supplementation in OVX mice resulted in down-regulation of ERα mRNA levels in both cochleae and inferior colliculi compared to the non-primed OVX mice. ERβ mRNA levels were not affected by estrogen replacement therapy. This suggests that circulating hormones modulate ERα mRNA expression in the central and peripheral auditory structures examined, whereas ERβ mRNA levels are independent of circulating sex hormones. It could be hypothesized that ERα constitutes an interface for estrogenic regulation in the auditory system under normal conditions, whereas ERβ in the auditory system of mice is not directly dependent on circulating estrogens but is rather constitutively expressed. The fact that ERβ expression is independent of hormone levels, does not mean that it is of less importance for maintaing hearing ability. It was previously shown that ERβ is constitutively expressed to maintain hearing ability, and genetic ablation of ERβ accelerates age-related hearing loss (Simonoska et al., 2009b).

Increased estrogen levels during pregnancy have been previously correlated with down-regulation of ERs in the cochlea of rats (Simonoska et al., 2009a). In the inner ear of non-mammalian vertebrates (fish *A. burtoni*) sex steroid receptor mRNA expression is also negatively correlated with circulating estrogens (Maruska and Fernald, 2010). The latter study demonstrated a down-regulation of ERα and ERβa in

the inner ear of *A. burtoni* in high estrogen conditions, whereas circulating sex hormones did not regulate ERβb expression, suggesting that the latter may be of importance for the continuous maintenance of auditory function and inner ear homeostasis. These results further support the notion that individual regulatory mechanisms and distinct physiological functions exist for each ER subtype with common elements across species.

Screening of changes in the cellular RNA pool of estrogen receptors was combined with evidence of estrogen-dependent modulation in auditory-related behavioral responses. The alterations in the behavioral measures in the OVX+E2 group (increased PPI and increased T_{MAX} in the between-group-comparisons, in hand with a highly significant effect of block on ASR magnitude during the session) indicate that 17β-estradiol replacement therapy results in a finer control of the startle response in mice. Lower structures of the auditory pathway, including the cochlear nucleus and inferior colliculus are known to be major components of these behavioral responses (Davis et al., 1982, Koch, 1999). No causal effects between the molecular and behavioral changes induced by 17β-estradiol treatment have been proven herein. Nevertheless, the concurrence of down-regulation of ERα in the cochlea and inferior colliculus and the improvement in the control of ASR and PPI with chronic 17β-estradiol treatment, suggests a possible link between circulating levels of estrogens and an ERα-mediated improvement of the startle response. A refinement of neurotransmitter activity mediated by ERα located in the cochlea and IC could be hypothesized. The neuromodulatory role of estrogens on sensory processing and autonomic responsiveness through interaction with dopamine and other neurotransmitters such as GABA, glutamate and serotonin is well established (Balthazart et al., 2006). The observed increase in PPI could relate to an improvement of dopaminergic activity in these mice due to a neuroprotective effect of estrogen and their receptors.

However, the neuronal circuit contributing to the ASR and PPI includes both auditory and non-auditory structures. Therefore, acoustic startle response and the behavioral measures related to it, is a system-wide outcome to which both auditory and non-auditory systems have significant contributions. Herein we have concentrated on the auditory component of the startle response rather than the attentional, stress-related or emotional aspects of it. Nevertheless, estrogen receptors are widely expressed in brain areas such as the hippocampus and amygdala of the limbic system, which are tightly connected to emotions, attention and learning (ter Horst, 2010). Thus, chronic estradiol treatment may also have significant effects on these components of the startle response. Defining the degree of involvement of brain regions related to attention, stress and learning is needed in order to fully appreciate the estrogenic effects on these behavioral measures.

No statistically significant differences were found between the sham-operated and the ovariectomized mice. The heterogeneity in the hormonal profile of intact animals, could partly account for this fact. The reason why these animals were not controlled for the phase of estrous cycle was to avoid differences in handling of the animals prior to the behavioral experiments, which may influence the behavioral outcome thereafter. Technical difficulties precluded accurate measurement of estrogen levels of the mice used in our study, therefore the exact hormonal profile of each mouse is not known.

We should keep in mind that by removing the ovaries we deprive the animals of their main source of estrogens but estrogen production is still present in other tissues of the body than the ovaries, including the brain. These estrogens may act locally in the brain in a paracrine way. In order to assess the effects of total depletion of estrogens one should examine what happens when aromatase expression is blocked and thus

estrogen production is completely inhibited. The reason why we used in our paradigm OVX as a condition of low estrogen levels is because it is clinically more relevant to human pathologies (pharmacological or surgical castration and hormone replacement therapy, menopause). Furthermore, estradiol acts not only via ERs and gene transcription, but also similar to a neurotransmitter through fast non-genomic pathways that modulate neuronal function within seconds or minutes (Balthazart and Ball, 2006). The impact of rapid non-genomic pathways of estrogens on auditory processing is largely unknown and need to be investigated.

A number of studies have demonstrated estrogenic effects on auditory processing with widely different levels of complexity and complementary perspectives (molecular, physiological and behavioral). For the interpretation of the latter, higher cognitive components of auditory processing and their interactions with the emotional and attentional components of hearing perception and behavioral outcome need to be encountered. Nevertheless both the correlation and the causal relationship between molecular changes and the physiological or behavioral outcome are steps of great value in the effort to understand estrogenic effects on the auditory system.

5.4 ERβ- AND BDNF-MEDIATED PROTECTION AGAINST ACOUSTIC TRAUMA

Genetic ablation of each estrogen receptor type and aromatase gave valuable insight in the role of estrogens and their receptors in auditory physiology. Normal hearing ability and cochlear morphology in all knock-out mice revealed that estrogen receptors are not critical in retaining normal function under physiological conditions. This is in agreement with another study showing differences in inner ear morphology of aged, but not young (3 months old), ERβ-deficient mice (Simonoska et al., 2009b). The introduction of a challenge of the auditory system activated an ERβ-mediated protective mechanism. Increased susceptibility of BERKO and ARKO mice compared to ERKO and WT highlighted the role of ERβ in hearing protection against acoustic trauma. The role of ERα was proven to be less important for this physiological procedure. Pre-treatment with ERβ-selective agonist (DPN) prior to acoustic trauma protected auditory ability of ARKO female mice compared to WT to a larger extent than ERα-selective agonist pre-treatment. This further highlighted the role of ERβ rather than ERα in the estrogenic effects on protection against acoustic trauma. Female mice were tested randomly with respect to their reproductive cycle, and this may be partly responsible for the lack of sex differences. However, constitutive expression of ERβ in the cochlea, uninfluenced by 17β-estradiol treatment or estrous cycle, which was demonstrated in paper III and our preliminary results, favours the sex-neutrality of ERβ effects in the cochlea.

Lower expression of BDNF in the cochlea of BERKO and ARKO mice compared to WT mice, accompanied susceptibility of these mice to acoustic trauma. DPN-induced up-regulation of BDNF protein expression in ARKO and ERKO mice, as well as BDNF mRNA expression in WT mice further confirms the ERβ-mediated modulation of this neurotrophic factor in the cochlea. Previous reports showing dimished hearing ability, hair cell loss and primary auditory neuron loss in BDNF knockout mice (Ernfors et al., 1994, Agerman et al., 2003) and protection of auditory neurons from neomycin toxicity by BDNF gene therapy (Staecker et al., 1998), are in accord with protective effects of BDNF demonstrated in the present study. Furthermore, these results provide a link between BDNF effects and estrogenic activity via ERβ. This could either happen through direct activation of BDNF transcription through the estrogen respomse element located in the gene encoding BDNF (Sohrabji et al., 1995) or via indirect mechanisms, similar to those uncovered in the cortex

(Blurton-Jones et al., 2004). Estrogen-BDNF interactions have been previously described in a number of neurodegenerative diseases (Sohrabji and Lewis, 2006). The ERβ-BDNF protection against acoustic trauma demonstrated here, highlights the potential of specific estrogen receptor modulators and BDNF as protective agents against decline of auditory function and degeneration of auditory neurons and hair cells caused by acoustic trauma.

5.5 ESTROUS CYCLE AND THE AUDITORY SYSTEM

The assessment of ERs expression in the cochlea during the estrous cycle, yields results similar to those obtained in paper III, as far as the impact of circulating estrogen levels on ER expression levels is concerned. Down-regulation of ERα mRNA levels in the cochlea was observed in proestrous, which is the phase of the reproductive cycle when the estrogen surge takes place. No effect of the estrous cycle was shown on ERβ mRNA expression. Modulation of ERα but not ERβ in the cochlea by hormonal levels, suggests that ERα is more relevant to changes in auditory function observed during the estrous cycle. The lack of a tight correlation between hormone levels (and thereby endocrine activity) and ERβ expression in the examined auditory structures, suggests that ERβ may be constitutively expressed in the auditory system, serving basic components of hearing or protecting hearing against challenges, such as the acoustic trauma.

Higher levels of the acoustic processing, such as perception of acoustic meaning related to maternal motivation behaviour, or other auditory components which bear reproductive relevance and value, are affected during the reproductive cycle (Koch and Ehret, 1989, Ehret and Schmid, 2009, Miranda and Liu, 2009). These changes are generally attributed to biological changes in higher centers of the auditory processing, which manage integrated information from several systems. Our preliminary results suggest that estrous cycle-dependent changes in acoustic perception may involve the periphery of the auditory system as well. Subtle changes in auditory processing during the reproductive cycle have been detected by a number of audiological tests (Haggard and Gaston, 1978, Elkind-Hirsch et al., 1992, Serra et al., 2003, Walpurger et al., 2004, Al-Mana et al., 2010), including hormonal influence of the lower, reflexive auditory functions, for example decreased prepulse inhibition in the luteal compared to the follicular phase (Swerdlow et al., 1997, Jovanovic et al., 2004). Results from a study in rats further confirm these data from human studies (Koch, 1998). However, controversial results showing stable ASR and PPI during the reproductive cycle, come from different strains of mice and rats (Plappert et al., 2005, Meziane et al., 2007, Adams et al., 2008). Our results suggest that these reproductive cycle-related functional changes of the auditory system, may be partly explained by modulation of ERα levels in the cochlea. Further investigation is needed in order to reveal the precise functional role of ERα modulation in the cochlea, and the causality between biochemistry and auditory function. Down-regulation of ERα in the cochlea, suggests that estrogens may affect the processing of sensory input and the auditory function in more peripheral locations than previously thought. This way estrogens could fine-tune our perception of the world already from the level of reception and transduction of signals in the cochlea, in order to match the needs of the rest of the body.

6 CONCLUSIONS

Wide expression of estrogen receptors throughout the auditory system and changes in ERs expression in auditory structures across different ages suggest that the auditory system is much more estrogen-responsive throughout life than previously thought. Regulation of ERα expression in central and peripheral auditory structures by chronic 17β-estradiol treatment and by fluctuating hormone levels during the reproductive cycle are both indicative of the role of ERα as an interface between the auditory system and circulating sex hormones. This is likely enabling the auditory system to fine-tune its' function in response to the hormonal milieu. Auditory-related behavioral changes concurrent with changes in ERα transcription in the auditory system induced by chronic 17β-estradiol treatment suggest a refining functional role of estrogens in auditory processing. Causality between biochemical and functional changes in the auditory system needs to be further investigated. Furthermore, ERβ-mediated neuroprotective mechanisms against noise exposure involving neurotrophic factor BDNF are suggestive of estrogens' supportive contributions to the auditory function. Thus, estrogens are likely to affect multiple structures of auditory processing throughout life, by fine-tuning basal auditory functions through alpha receptors and by protecting against acoustic trauma through beta receptors. Taken together, the results presented in this thesis point towards an involvement of estrogens and their receptors in auditory physiology. Unwrapping the estrogen-auditory system interactions may present new treatment strategies and novel pharmacological targets for the support of hearing.

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