Thesis for doctoral degree (Ph.D.) 2009

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SEX STEROID HORMONE RECEPTORS

INNER EAR & HEARING

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STOCKHOLM 2009

Published by Karolinska Institutet.

ISBN 978-91-7409-480-0

Printed by


www.reproprint.se
Gårdsvägen 4, 169 70 Solna

“In the middle of difficulty lies opportunity”

Albert Einstein

ABSTRACT

It is well known that hearing loss is more profound in elderly males than females, regardless of noise exposure. Also, an age-related hearing decline starts much earlier in males, already after the age of 30, while in women, the decline does not start until the age of 50. This coincides with the menopausal transition in most women, when endogenous circulating levels of estrogen in the body are reduced. It is also well known that women with Turner syndrome who are estrogen deficient suffer from early onset of age-related hearing loss already at the age of 35.

The overall aim of this thesis was to investigate whether the female sex steroid hormone, estrogen, and its receptors are important in maintaining hearing. The effects of estrogen, estrogen-modulators and anti-estrogen on the estrogen receptors (ERs) in the inner ear were investigated. The expression pattern of estrogen receptors during periods of maximum and minimum hormonal levels such as pregnancy, maturation and development was characterized. The experimental studies were performed using rat and mice animal models.

We found that ERs are up and down-regulated in the inner ear depending on the stage of maturation, development and pregnancy. In post-natal rats, ER expression levels appear to be inversely correlated to estrogen levels (e.g. when high levels of estrogen are present, ERs are expressed less in the inner ear). At the time points measured in non-fetal rats, ERα and ERβ expression was highest in three-week-old rats (a known period of fairly low estrogen levels). ER expression reached the lowest levels in pregnant mother rats in late pregnancy (a known period of high estrogen levels). There was no ERα or ERβ identified in the inner ears of the fetuses, at either E8 or E18. No estrogen receptors were found in the cochlea of the developing fetus. These findings suggest that estrogen may have an effect on the cochlea during various stages of life, but seems not to be active during gestation.
Treatment of ovariectomized rats with estradiol, tamoxifen, anti-estrogen or vehicle only, did not alter the ER expression pattern significantly in the inner ear. However, a slight down regulation of ERα in the marginal cells of the stria vascularis (involved in ion regulation) was seen in rats that were injected with pure anti-estrogen, suggesting that ERα may be involved in the ion regulation in the cochlea.
When investigating estrogen receptor β deficient mice, altered inner ear morphology was found, corresponding to deafness already by one year of age. Also, both ERs are present in the inner ear of wild type (WT) mice at specific localizations suggesting subtype-specific functionality.

All together the findings of this thesis strengthen the hypothesis that estrogen has a direct effect on hearing functions and may imply that ERβ is important for the prevention of age-related hearing loss. This study provides a better understanding of the observed positive hearing effects of hormone replacement therapy in patients with low estrogen levels (e.g. postmenopausal women).
Several interesting areas for further investigation arose from the work of this thesis including: the protective role of ERβ on hearing along with its possible interactions with ERα; the complex interactions of the reproductive hormones in the inner ear along with their effects on target organ morphology and hearing, and localization and functionality of other reproductive hormone receptors.

LIST OF PUBLICATIONS

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

I. **Simonoska R**, Stenberg AE, Masironi B, Sahlin L and Hultcrantz M. (2009) Estrogen receptors in the inner ear during different stages of pregnancy and development in the rat. *Acta Oto-Laryngologica Feb 13: 1-7.*

II. Stenberg AE, **Simonoska R,** Stygar D, Sahlin L and Hultcrantz M. (2003) Effect of estrogen and antiestrogens on the estrogen receptor content in the inner ear of ovariectomized rats. *Hearing Research 182: 19-23.*

III. **Simonoska R**, Stenberg AE, Duan M, Yakimchuk K, Fridberger A, Sahlin L, Gustafsson JÅ and Hultcrantz M. (2009) Inner ear pathology and loss of hearing in estrogen receptor beta deficient mice. *Journal of Endocrinology. In press.*

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ABBREVIATIONS

ABR	Auditory brainstem response
ANOVA	Analysis of variance
AR	Androgen receptor
BSA	Bovine serum albumin
CN	Cranial nerve
Cy5	Indocarbocyanine
dB	Decibel
DPOAE	Distortion product otoacoustic emissions
ER	Estrogen receptor
ERα-/-	Estrogen receptor alpha knock out
ERβ-/- (BERKO)	Estrogen receptor beta knock out
FITC	Fluorescein isothiocyanate
HRT	Hormone replacement therapy
IHCs	Inner hair cells
kHz	kilo Hertz
mRNA	Messenger ribonucleic acid
OHCs	Outer hair cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PR	Progesterone receptor
RT	Room temperature
sc	Subcutaneous (injection)
SERM	Selective estrogen receptor modulator
SGN	Spiral ganglion neuron
SPL	Sound pressure level
TBS	Tris-buffered saline
TS	Turner syndrome
WT	Wild type

1 INTRODUCTION

Sex steroid hormones govern many aspects of life. Besides the reproductive functions, sex hormones also affect many organs in the body such as connective tissue, hair follicles, muscles, brain, bone, skin and blood vessels. The sex steroid hormones have a well-known effect on brain function, and they possibly also affect brain aging (Wang et al., 2002; Brann et al., 2007). There are both animal and human studies indicating that the sex steroid hormones are active also in the hearing process (Coleman et al., 1994; Guimaraes et al., 2006; Meltser et al., 2008). An intriguing aspect is the gender difference in hearing, observed in humans and animals, where females have been shown to have better hearing than males (Guimaraes et al., 2004; Jonsson et al., 1998). The relationship between hearing, sex steroid hormones and their receptors is not fully understood. The overall purpose of this study is to further elucidate these relationships.

1.1 ANATOMY OF THE INNER EAR

The ears are sensory organs comprising both the auditory system, which is involved in the detection of sound, and the vestibular system, which is responsible for maintaining one's balance. The ear may be divided anatomically and functionally into three regions: the external ear, the middle ear, and the inner ear (Figure 1). All three regions are involved in hearing.

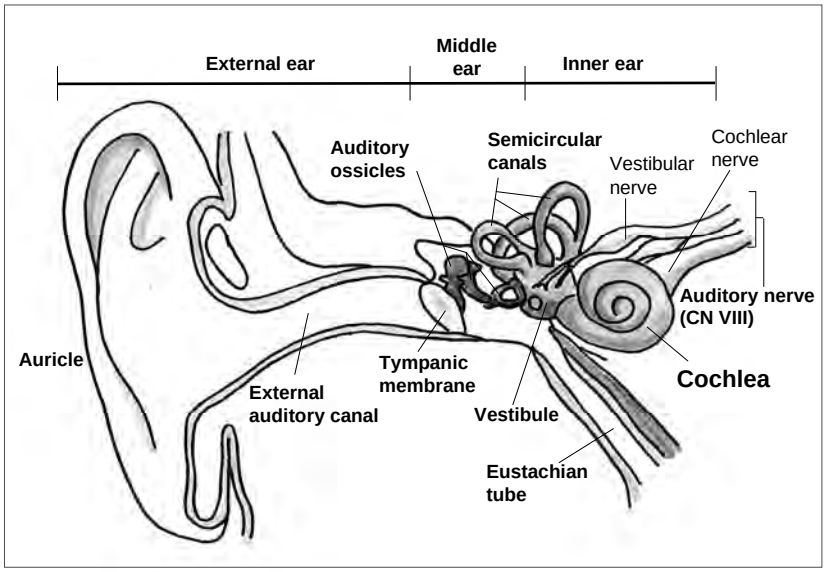

Figure 1. *Anatomy of the human ear. The external ear consists of the pinna (auricle) and the external auditory canal; the middle ear consists of the tympanic membrane, the auditory ossicles, the middle ear cavity and the Eustachian tube; the inner ear consists of the oval and round windows, the cochlea and the semicircular canals.*

The anatomy of the inner ear is dominated by large fluid-filled spaces, which consist of a maze of fluid-filled tubes. The bony tubes (i.e. the bony labyrinth) are filled with a fluid called perilymph. There are three major sections of the bony labyrinth: the snail-shaped cochlea (whose size and number of turns differ between species) whose function is hearing; the semicircular canals with their ampullae involved in balance; and the vestibule, which forms a conduit that connects the cochlea and the semicircular canals and contains the sense organs for acceleration, called the utricle and saccule.
Within this bony labyrinth of the inner ear there is a second series of delicate cellular tubes, called the membranous labyrinth. This membranous labyrinth contains the actual hearing cells. There are two main types of hearing cells: the inner and outer hair cells (IHCs and OHCs), and together with other supporting cells, they form the Organ of Corti. The Organ of Corti is seated on the basilar membrane (Figure 2).

The mouse cochlea has 1.75-2.5 turns, depending on mouse strain and fixation technique (Keiler et al., 2001; Burda at al., 1988); the length of its basilar membrane is approximately 6 mm (Keiler et al., 2001); and it contains about 700 inner hair cells and around 2400 outer hair cells (Bohne and Harding, 2004).

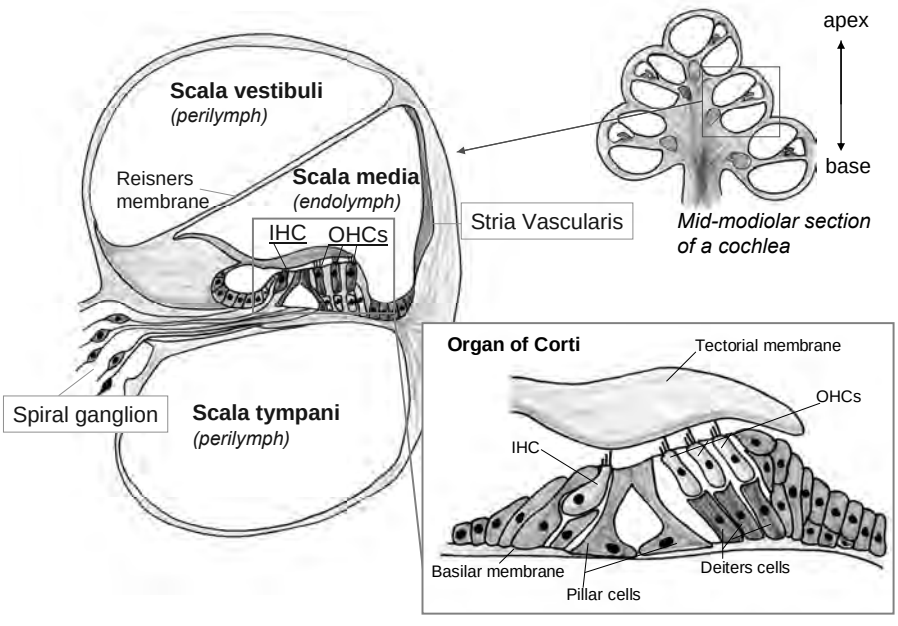

Figure 2. *Schematic drawing of the cochlear anatomy. Top right corner: mid-modiolar section of aa human cochlea. The left figure illustrates the spiral canal of the cochlea divided into three membranous ducts (scala vestibuli; scala media; and scala tympani, which spiral around the modiolus to the apex of the cochlea). The Organ of Corti is positioned in the scala media containing the inner and outer hair cells (IHC and OHCs) (bottom right corner).*

The spiral ganglion is localized in the Rosenthal canal, which is spirally arranged in the modiolus, also known as a central bony axis of the cochlea. It links the auditory receptors in the Organ of Corti with the brain stem. The peripheral processes of the spiral ganglion neurons extend to the hair cells in the Organ of Corti, while their axons

reach the cochlear nucleus and form a part of the eighth cranial nerve (CN VIII). There are two types of spiral ganglion cells: type I cells, also known as large cells and commonly represents the main population in the spiral ganglion (90-95%); and type II cells, also recognized as part of the small cells in the spiral ganglion, and usually represents about 5-10 % of the spiral ganglion population. While type I cells are myelinated ganglion cells that participate in the afferent innervations of the IHCs and therefore leading most of the afferent input in the brain stem, the type II cells are unmyelinated ganglion cells and they are believed to innervate the OHCs (Spondelin, 1979).

1.2 THE AUDITORY PATHWAY

From sound waves to actual perception, the air born sound waves are captured by the auricle; travel through the external auditory canal and initiate vibrations in the tympanic membrane. The pressure difference across the tympanic membrane causes it to vibrate and these vibrations are conducted through the auditory ossicle chain (malleus, incus and stapes) to the fluids of the cochlea. In the cochlea, movement of the fluid makes the basilar membrane, on which the sensory cells are located, vibrate. The sensory hair cells detect the mechanical vibration and convert it into electrical signals. These neuronal signals (impulses) initiated by the IHCs are conducted through afferent nerves with relay stations in the inner ear (spiral ganglion), in the brainstem (cochlear nucleus, superior olivary complex and inferior colliculus), in the thalamus (medial geniculate body) and in the temporal lob of the brain (auditory cortex) (Figure 3). In the auditory cortex, the impulses are translated into sensation known as sound.

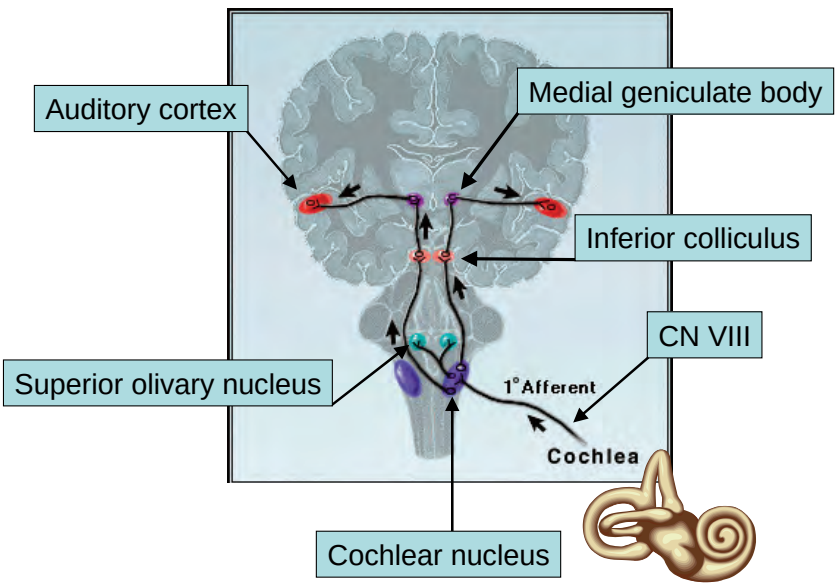

Figure 3. *The central auditory pathway. The electrical responses generated in auditory brainstem response (ABR) measurements travel along the brainstem auditory pathway through relay stations from the cochlea all the way to the auditory cortex.*

The hearing threshold in animals is detected by auditory brainstem response (ABR) measurements. The different relay stations can be visualized and are responsible for generating the ABR-waves (Figure 4):

- ***Wave I*** is the far-field representation of the compound auditory nerve action potential in the distal portion of cranial nerve (CN) VIII. The response is believed to originate from the CN VIII fibers as they leave the cochlea and enter the internal auditory canal.

- ***Wave II*** is generated by the proximal part of the CN VIII as it enters the brain stem.

- ***Wave III*** arises from the neuron activity beyond CN VIII, in or near the cochlear nucleus. Literature suggests wave III is generated in the caudal portion of the auditory pons.

- ***Wave IV*** often shares the same peak with wave V, and is thought to arise from the pontine neurons that are mostly located in the superior olivary complex, but additional contributions may come from the cochlear nucleus and nucleus of lateral lemniscus.

- ***Wave V*** likely reflects activity of multiple anatomic auditory structures. The ABR wave V is the component analyzed most often in clinical applications of the ABR. Generation of wave V is believed to originate from the vicinity of the inferior colliculus.

- ***Wave VI*** and ***VII*** are thought to be generated in the thalamus (medial geniculate body), but the actual site of generation is uncertain (Markand et al., 1994).

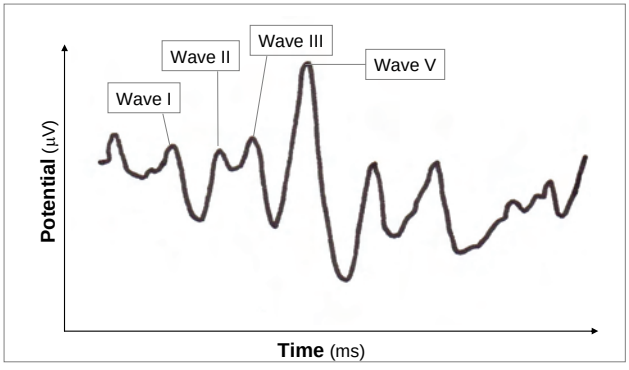

Figure 4. *The Auditory Brainstem Response (ABR), which originates in the VIIIth cranial nerve (waves I and II) and brainstem auditory structures (wave V: region of lateral lemniscus and inferior colliculus).*
The horizontal axis represents the time in ms (milliseconds), and the vertical axis represents the amplitude in μV (microvolt).

Prolonged latencies (more time needed for same sound to propagate) indicate that there is a delay of the impulses in the cochlear nerve and in the flow of information through the central hearing pathways to the hearing centers of the brain. Prolonged latencies are a sign of suspected retrocochlear pathology (e.g. acoustic neuroma or vestibular

schwannoma), but can also be altered by hormonal influences. For example, in ovariectomized rats (i.e. removal of the endogenous estrogen production), ABR measurements show prolonged latencies. These changes are reversed by estrogen replacement (Coleman et al., 1994).

1.3 STEROID HORMONES

There are five major classes of steroid hormones:

- estrogens
- androgens
- progestogens (=gestagens)
- mineralocorticoids
- glucocorticoids

Steroid hormones are known to act in, among other tissues, neural tissues affecting brain development and behavior. These actions are known to be mediated by specific intracellular receptors since the brain contains receptors for all of the five classes of steroid hormones (Jung-Testas et al., 1992).
They are all derived from cholesterol (Figure 5), which consists of 27 carbons. Three of the classes of steroid hormones are sex steroid hormones: progestogens (21 carbons), androgens (19 carbons) and estrogens (18 carbons).

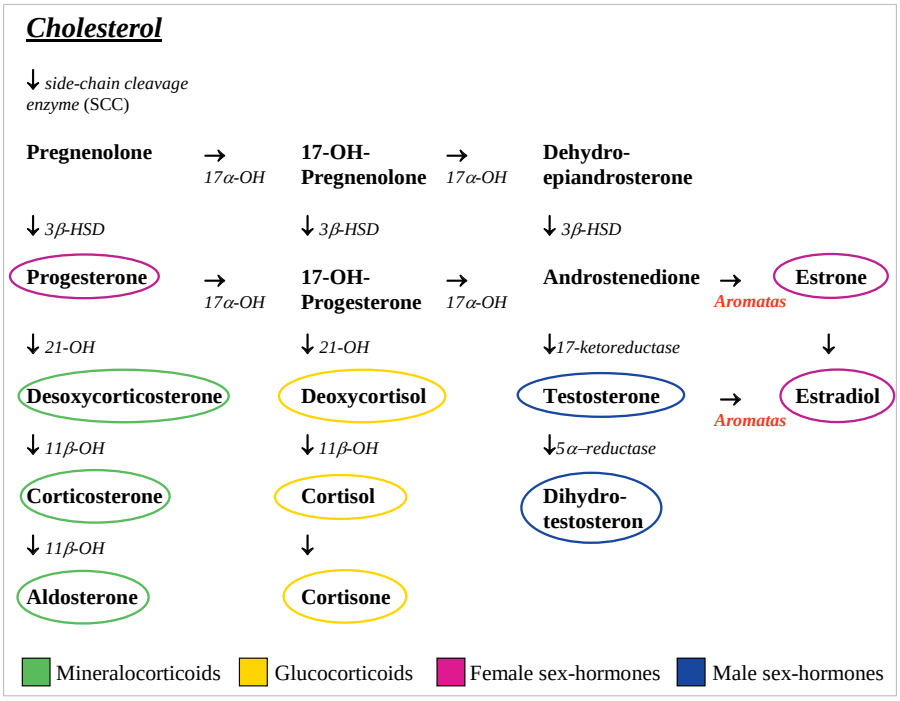

Figure 5. *Steroid hormone synthesis.*

1.3.1 Sex steroid hormones and their receptors

Sex steroid hormones are also known as *gonadal hormones*. The majority of natural sex steroid hormones are produced by the gonads (ovaries or testes). Their effects are mediated by slow genomic mechanisms through nuclear receptors, as well as by fast non-genomic mechanisms through membrane-associated receptors and signaling cascades.
Sex hormones play important roles in inducing the development of primary- and secondary sex characteristics in the body. In general, estrogens and progestogens are considered "*female sex hormones*" while androgens are considered "*male sex hormones*" since they have feminizing and masculinizing effects respectively, (ElAttar and Hugoson, 1974). All types are present in both gender but at different levels.

Androgens

Androgens (testosterone [T] and 5α-dihydrotestosterone [DHT]) control the development, differentiation, and function of male reproductive and accessory sex tissues, such as the seminal vesicle, epididymis, and prostate. Other organs and tissues, such as skin, skeletal muscle, bone marrow, hair follicles, and the brain, are also under the influence of androgens. The primary mechanism of action is either activation of androgen receptors (AR) or aromatization to estradiol, then activation of estrogen receptors. Both lead to changes in gene expression and protein synthesis (Davison et al., 2006; Lu et al., 2006). The androgen receptor exists in a full length B-form (AR-B) weighing 110 kDa and a shorter A-form (AR-A) weighing 87 kDa. AR-A has a truncated N-terminus (lacks the first 187 amino acids) (Wilson et al., 1994).

Estrogens

The main source of estrogen production is the ovaries, but a small amount also originates from other tissues such as the adrenal glands, adipose tissue and brain. In these secondary tissues, the production occurs via the conversion of the male hormone androgen to estrogen by aromatization. The effect of the endogenous estrogen (17β-estradiol) is exerted in a genomic way through estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). There are also short-term non-genomic effects primarily in the central nervous system (Wierman et al., 2007), where estrogen has been claimed to bind to ERs localized on the plasma membrane of target cells (Nelson et al., 1986; Razandi et al., 1999; Razandi et al., 2004). The non-genomic effects are typically faster than the genomic action, the former occurring within minutes rather than hours (Lee and Marcus, 2001).
The cDNA encoding an ER protein was first cloned and described 1984 (Green et al., 1984; Greene et al., 1984). The name changed to ERα when ERβ was described in 1996 (Kuiper et al., 1996). ERβ is partially homologous to the ERα especially in the DNA binding domain (97% amino acid identity) and ligand binding domain (59% amino acid identity) (Enmark et al., 1997). Even though ERα and ERβ have similar binding affinity to estradiol, and they share many common functional characteristics, their molecular and transcriptional activity and the tissue localization are different (Kuiper et al., 1997). The ERα is found in the brain, the breast, the liver, the

cardiovascular system, the reproductive tract, bone and in the urogenital tract; while ERβ is found in the heart, kidney, brain, lungs, the reproductive tract, gastrointestinal tract, bone and the urogenital tract (Koehler et al., 2005; Karas et al., 1994; Saunders et al., 1997; Taylor and Al-Azzawi, 2005). Both ERα and ERβ are found in the inner ear of humans, rodents and fish (Stenberg et al., 2001; Stenberg et al., 1999; Forlano et al., 2005).

Progestogenes

Progesterone is the naturally occurring progestogen and a subclass of the sex hormones. Progesterone is known as the hormone of pregnancy; it prepares the endometrium for implantation, keeps the myometrium quiescent until parturition and affects the female immune system to accept the fetus. In addition to having important hormonal effects, progesterone serves as a precursor in the synthesis of estrogens, androgens and adrenocortical steroids. It is synthesized in the ovary, testis, and adrenal gland from circulating cholesterol. Large amounts are also synthesized and released by the placenta during pregnancy. Synthetically synthesized progesterone-like substances called progestogens or progestins, are used in pharmaceuticals, such as oral contraceptives and hormone replacement therapy (HRT).
Progesterone effects are mediated by the binding to the progesterone receptors (PR). PR is expressed in two protein isoforms, PRA and PRB, arising from the same gene due to use of different promoters, thus creating two separate mRNAs. PRA is identical to PRB apart from lacking 164 amino acids from the N-terminal (Conneely et al., 2003).

All sex steroid hormone receptors are members of the nuclear receptor super-family, which also includes steroid-, thyroid-, vitamin D-, and retinoic acid receptors. The action of steroid hormones has been thought to occur by mRNA transcription and protein synthesis via nuclear genomic action (Figure 6), (Norman and Litwack, 1997). However, numerous non-genomic effects have been described in various tissues and species and in all groups of steroids (Edwardson and Bennet, 1974; Pietras and Szego, 1975; Spach and Streeten, 1964).

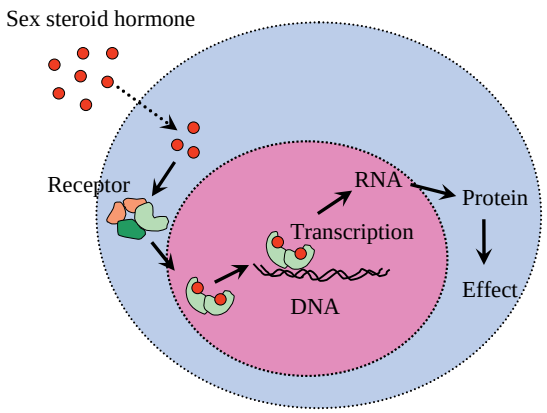

Figure 6. *The activation of sex steroid hormone receptors. After the entrance in the cell by passive diffusion, the hormone binds to the intracellular receptor, which in its inactivated state exists in either the cytoplasm or the nucleus. After the binding, the sex hormone receptor forms a dimer, which binds to DNA. That leads to activation of the transcription process and synthesis of a specific messenger RNA and protein production.*

1.3.2 The presence of sex steroid hormone receptors in the inner ear and the effect on hearing

It is known from earlier studies that the two subgroups of estrogen receptors (ERα and ERβ) are present in the inner ear of humans, rodents and fish at locations important for sound transmission (Stenberg et al., 2001; Stenberg et al., 1999; Forlano et al., 2005). Estrogen and both receptor subgroups seem to be active in the hearing process (Coleman et al., 1994; Meltser et al., 2008). On the other hand, the presence of progesterone or androgen receptors in the inner ear has not yet been verified, however, it has been shown in a study by Sisneros et al that non-reproductive (ovariectomized) female midshipman fish, supplemented with testosterone and estrogen, exhibit an increase in auditory sensitivity (Sisneros et al., 2004). In the nervous system, it is known that the presence of progesterone receptor has been found in Schwann cells (Jung-Testas et al., 1996) and progesterone has been shown to modulate cell growth and the synthesis of myelin proteins in rat glial cells of the central nervous system (Jung-Testas et al., 1992). Keast and Gleeson, showed the presence and distribution of AR in primary afferent neurons in the dorsal root ganglia of rat (Keast and Gleeson, 1998).

Gender difference

Humans

Unlike women, the hearing in men starts to decline in the high frequency ranges between age 20 and 30. Hearing in women is fairly constant up to 40-50 years of age, when they normally enter menopause (Pearson et al., 1995). This seems to be a critical time for hearing loss in women, when the hearing thresholds are rapidly increasing (i.e. the hearing deteriorates). Thereafter the hearing loss progresses, but at a slower pace and resembles the rate of decline seen in males (Hederstierna et al., 2007). In addition, women with Turner syndrome (total or partial loss of one X chromosome) who are biologically estrogen deficient have longer ABR latencies and an earlier onset of age-related hearing loss compared to other women (Gungor et al., 2000; Hultcrantz et al., 1994). The lack of endogenous estrogens has been proposed to be a contributing cause for the sensorineural high-frequency hearing loss in women with Turner syndrome (TS). Estrogens have neuroprotective and neurotrophic effects on the brain (Brann et al., 2007), and therefore presumed to have positive effect on the hearing. Moreover, it is well documented that the time for sound transmission from the inner ear to the auditory centre in the brain is shorter in women than in men, as measured by ABR (Jerger and Hall, 1980). Also, there is a greater difference in ABR latencies in younger vs. older women (postmenopausal) compared to age related differences in men (Jerger and Johnson, 1988; Rosenhamer et al., 1980; Warton and Church, 1990). These gender differences can not solely be explained by anatomical variations or occupational noise (Jonsson et al., 1998).

Estrogen seems to play a protective role on the hearing organ, and a lack of estrogen seems to accelerate age related hearing loss and also affect ABR threshold, which is seen in menopausal women with HRT that have better hearing than untreated women (Hederstierna et al., 2007; Kilicdag et al., 2004). Despite the evidence of a protective effect of estrogen, there are case reports that hormone replacement therapy and oral contraceptive use can lead to acute sudden deafness (Hanna, 1986; Strachan, 1996), and

the symptoms in women with Ménièr's disease, (episodic symptoms of rotational vertigo, low frequency hearing loss, aural fullness and tinnitus), seem to get worse during the premenstrual phase, when the estrogen levels are low (Andrews et al., 1992). Contradictory effects of estrogen have been observed in several systems in the body and may partly be due to the fact that estrogen action depends on the balance between activation of two estrogen receptors, ERα and ERβ which in some situations, can oppose each other's actions (Kushner et al., 2000). It could also partly be explained by the possible influence of progesterone or androgens on the inner ear, and their balance with estrogen. Guimaraes, et al showed that the presence of progestin, as a component of HRT, results in poor hearing abilities in aged women (Guimaraes et al., 2006). However, very limited investigations have focused on the effect of progesterone and androgens on the inner ear and hearing.

Animals

In experimental animal models it has been shown that non-reproductive (ovariectomized) female midshipman fish, supplemented with estrogen or testosterone implant, exhibit an increase in auditory sensitivity (Sisneros et al., 2004). There is also evidence indicating hormonal effects on presbyacusis in mice, where in particular circulating estrogen levels have effects on otoacoustic emissions (OAEs) (Thompson et al., 2006). Other studies have shown that middle-aged female CBA mice have healthier outer hair cells (OHCs) compared to middle-aged males, as measured by distortion product otoacoustic emissions (DPOAE), but after menopause these advantages for the female mouse are lost. No sex difference was observed for young mice (Guimaraes et al., 2004). In humans, the sex difference in OAEs is evident at birth and persists through life (McFadden, 1998).

ER knock out mice has been used for studying the effect of the estrogen receptor on the hearing. In young (3 months) ERβ knock out mice (BERKO or ERβ-/-), the inner ear appeared to be normal, except for swollen afferent nerve endings (Stenberg et al., 2002), and when exposed to acoustic trauma these mice were more vulnerable to hearing loss than their wild type (WT) littermates (Meltser et al., 2008). It is also known that in the brain of the ERβ-/- mice, with increasing age, neuronal loss is present (Wang et al., 2001).

Knock-out mice as model for hearing research

With the development of gene technology, it is now possible to manipulate specific target genes. The development of the different sex steroid hormone receptor knock-out mice has provided models to study the function of these nuclear receptors in different organs in the body that they may affect, e.g. the inner ear.

The generation of ERβ-/- mice (used in Paper III) has previously been described by Krege et al. (Krege et al, 1998). Ablation of both ERs, or selective deletion of ERα or ERβ indicated distinct biological roles of the receptors in other mouse tissues, described in: mammary gland (Tekmal et al., 2005), uterus (Couse et al., 1999), ovaries (Krege et al., 1998; Dupont et al., 2000), prostate (Prins et al., 2001), cardiovascular system (Pare et al., 2002), brain (Dubal et al., 2001; Wang 2001), bone (Windahl et al., 2002), skeletal muscle (Brown et al, 2009), kidney (Lane, 2008), neurobehavioral studies (Choleris et al., 2008) and anxiety regulation (Tomihara et al., 2009).

The reason of using ERβ-/- mice in this thesis was the previous studies done in brain showing an early onset of age-related degeneration, expressed by the significant neuronal loss (Wang et al., 2001). The authors concluded that ERβ is necessary for neural survival in the brain. We wanted to test if the same hypothesis also applies for the inner ear morphology and investigate the effect a lack of ERβ could have on the hearing function.

1.4 STAINING DIFFICULTIES IN INNER EAR TISSUE

Due to the anatomical position of the inner ear well embedded in the temporal bone it is difficult to gain access to the organ and thereby complicated to use in immunohistochemistry staining. Research on human inner ears is therefore not possible without causing damage. This makes the use of animal models for research necessary.
Also, the short life cycle of the rodents makes it very suitable for studies of both embryologic development, but also of age-related hearing impairment. Even though animals are good models for research studies, the inner ear, and the hearing organ in particular, is embedded in hard bony structure, which makes the dissection and the preparation before application of immunohistochemistry and immunofluorescence procedures a delicate work. Compared to other organs, e.g. uterus and ovaries, the inner ear, especially in rodents, is not only a small structure, but also surrounded by bony structures, that needs to be decalcified, a step that other soft tissues does not need to go through.

The difficulty continues when considering the involved procedures required retrieving antigens when staining for sex steroid hormone receptors.
That, together with the issues of tissue-preparation makes it harder to obtain quality stains of the inner ear structures compared to other, soft, tissues.

1.5 RAT PREGNANCY AND INNER EAR DEVELOPMENT IN PUPS

Because steroid hormone receptors are inducible proteins, they may be present or absent in cells under various conditions. Such hormonally driven conditions are, among others, maturation, pregnancy and fetal development.

As a rat animal model the Sprague-Dawley rat strain was used (Paper I and II). The Sprague-Dawley rat is a well-characterized strain and has a life span of 2.5 -3.5 years. Estrous cycles normally begin after 54 days of age, and the rat is considered to be sexual mature at this age (Gabriel et al., 1992). Breeding onset occurs between 65-100 days of age in both females and males, although females may have their first estrus as early as at day 35. The average gestation period is 22 days, and a normal litter size is 6-12 pups. When the pups are born, the hearing organ is morphologically mature, but the ear canal is closed. Hearing is not fully developed until pups are 14 days old when the ear canal opens (Mikaelian and Ruben, 1964; Evans, 2005).

The levels of estrogen over the lifetime of the female rat have been well described. The estrogen level in the blood is low in young rats, and during puberty, the estrogen production in the female rat increases. During rat pregnancy, estrogen starts to increase on day 14 with a progressive rise continuing until delivery (Taya et al., 1981).

2 AIMS

The overall aim of this thesis was to investigate the importance of the sex steroid hormone estrogen, its receptor's expression pattern in the inner ear and its effect on hearing by using animal models at different time points in life.
In specific:

- To determine the expression pattern of estrogen receptors in the cochlea during pregnancy, maturation and development in a female rat model.

- To investigate the impact of estradiol, selective estrogen receptor modulators (SREMs) and anti-estrogen treatment on the expression levels of estrogen receptors in the cochlea of ovariectomized rats.

- To test the hypothesis that ERβ is important for preserving the inner ear morphology and hearing in ERβ knock-out mice.

3 MATERIALS AND METHODS

3.1 SPECIMENS

3.1.1 Animals (I-III)

In this thesis, both rat and mouse animal models were used. All studies (I-III) were approved by the local animal ethics committee at Karolinska Institutet.

All animals were housed in a controlled environment at 20°C on an illumination schedule of 12h of light and 12h of darkness each day. Standard pellet food and water were provided *ad libitum*.

Rats

In paper I and II inbred Sprague Dawley rats were chosen as a model for the studies used in order to decrease the effect of possible animal-to-animal variations. The pregnant rats used in Paper I, were delivered at the animal department on day 4 of pregnancy together with the non-pregnant rats. Fetuses (embryonic/gestational day 8 and 18) examined in this study were obtained for analysis when the pregnant rats were sacrificed.
Ethical permission no.: N359/02 and N200/05.

Mice

The mice used in paper III were deficient of estrogen receptor β, also called: estrogen receptor β knock-out mice (ERβ-/- or BERKO), and compared to their wild type littermates (WT). The ERβ-/- mice were created from two inbred mouse strains, C57BL/6J and J129. The ERβ-/- colony was maintained with heterozygous breeding after backcrossing ERβ+/- mice with C57BL/6J mice from Jackson Laboratories (Bar Harbor, Maine, USA) for more than 11 generations (Krege et al., 1998). All mice were genotyped using PCR with DNA extracted from their tails at 2 weeks of age in order to determine whether they were WT or ERβ-/-.
Ethical permission no.: N95/01; 297/03 and N81/06.

3.2 ANIMAL EXPERIMENTS

3.2.1 Ovariectomy and hormone treatment (II)

In Paper II, the ovariectomy was performed, according to standard procedure, on all animals after being anesthetized with Hypnorm® (Janssen-Cilag, Saunderton, UK) and midazolam (Alpharma, Oslo, Norway). The rats were then housed for 14 days before initiation of hormone treatment. The hormone treatment lasted for 48 h. The control group received vehicle only (propylene glycol carrier with no hormone). The animals received the subcutaneous (sc) injections in the morning of days 1 and 2, and were

sacrificed on the morning of day 3. The drugs used in this study are presented in Table 1.

Table 1. Summary of the drugs used in the present study.

Drug	Dose	Administration	Source
17β Estradiol (E_2)	2.5 mg/day	sc	Sigma, St. Louis, MO, USA
Tamoxifen	500 mg/day	sc	Sigma, St. Louis, MO, USA
ICI 182780	500 mg/day	sc	Kindly donated from Dr. A.E Wakeling

sc = subcutaneous injection.

Tamoxifen = selective estrogen receptor modulator.

ICI 182780 = pure anti-estrogen that binds to estrogen receptors and block the binding site.

17β Estradiol (E_2) is an estrogen that binds with same affinity to both ERα and ERβ (Kuiper et al., 1997). It is also used as a part of HRT, together with progestin.

Tamoxifen, a non-steroidal anti-estrogen, is the oldest and most-prescribed selective estrogen receptor modulator (SERM). It is metabolized in the liver into active metabolites which have 30-100 times more affinity for the estrogen receptor than tamoxifen itself. These active metabolites compete with estrogen in the body for binding to the estrogen receptor. Tamoxifen is both antagonist and agonist of the estrogen receptor. Depending on the tissue type, SERMs demonstrate both estrogenic and anti-estrogenic properties, e.g. Tamoxifen is an antagonist in breast tissue while it acts as partial agonist on the endometrium and has been linked to endometrial cancer in some women (Gallo and Kaufman, 1997).

ICI 182780 is a steroidal anti-estrogen that differs from non-steroidal anti-estrogens, such as tamoxifen. It is bound by estrogen receptors with a high affinity, similar to that for estradiol and it is a "pure" anti-estrogen (i.e. although it binds to the estrogen receptor, it does not activate the receptor and therefore, it does not mimic any of the physiological effects of estradiol) (Wade et al., 1993).

3.2.2 Auditory brainstem response (ABR) (III)

To analyze hearing (auditory capacity), the mice (Paper III) were tested with auditory brainstem response (ABR) at age of 3 and 12 months. After anaesthetizing the mice with an intraperitoneal injection of ketamine (50mg/kg) and xylazine (10mg/kg), responses to sound stimuli were recorded with three subcutaneously placed stainless-steel needle electrodes. Body temperature of the animal was maintained at 38^{o}C with an isothermic heating pad. The stimulus signal was generated through Tucker-Davis (Florida, USA) equipment controlled by a computer and delivered by an earphone sealed to the ear channel via an ear tube. The tested frequencies were from 6.3 kHz to 40 kHz. The stimuli consisted of a band-pass filtered single full sine wave and the symmetrical signal was filtered with a one-third-octave filter (Bruel & Kjaer type 1612). The evoked response was amplified 100,000 times and averaged (2048 sweeps

in each run) in real-time. Stimuli were presented at intensities well above threshold and then decreased in 10-dB steps until the ABR waves disappeared. Threshold was defined as the lowest intensity (dB) at which measurable ABR waves were seen in two averaged runs. The limit of the equipment at each test frequencies was: 6.3 kHz at 110 dB; 12.5 kHz at 135 dB; 20.0 kHz at 135 dB; 40 kHz at 95 dB. When no hearing was measurable at these frequencies, a value 5 dB above the limit of the equipment was used in the statistical analysis. Mean and standard deviation were calculated. The electric response was generated along the brainstem auditory pathway, and the different waves represent activity in the relays from the cochlea to the inferior colliculus. Five wave peaks are usually registered and represent potentials elicited from the eighth nerve, the cochlear nucleus, contra lateral superior olivary nucleus, the contra lateral inferior colliculus, and an area in-between the superior olive and inferior colliculus. Latencies of ABR responses are measured in milliseconds (ms) from the stimuli to the first peak (peak I) and also between peaks I to III (see Figure 4, in the Introduction section).

3.3 TISSUE COLLECTION (I-III)

3.3.1 Animals

All animals were sacrificed by decapitation after they had been deeply anesthetized with Xylazin (Bayer AG, Leverkusen, Germany; 0.75mg/100g rat) and Ketaminol (Intervet AB, Boxmeer, Netherlands; 7mg/100g rat).

Immediately after the animals were sacrificed, the temporal bones were removed. Then the stapes was luxated, the oval and round windows opened. A small fenestra was made in the bony shell of the cochlear apex, and the left cochlea was gently perfused with 4 % paraformaldehyde and then immersed in the same solution for one hour. Specimens were decalcified for approximately two weeks in 0.1M EDTA. When soft enough for sectioning the inner ears were embedded in paraffin. Paraffin-embedded cochlea-tissues were cut into 5µm sections, placed on positively charged glass slides (SuperFrost Plus, Menzel-Glaser, Braunschweig, Germany) and dried at 38^{o}C overnight. Then the immunohistochemical staining procedures described below were applied.

For the morphological studies and the quantification of spiral ganglion cell density (Paper III), the right temporal bone of each mouse was fixed in 2% glutaraldehyde and prepared according to standard methods for morphological analysis, i.e. embedded in JB-4 plastic (Polysciense Inc, Awrrington, PA, USA) and sectioned in 4µm sections. The sections were then mounted on glass slides and stained with Paragon.

3.4 IMMUNOHISTOCHEMISTRY (I-III)

3.4.1 Estrogen hormone receptors

A standard immunohistochemical technique (avidin-biotin-peroxidase) (Figure 7) was carried out to visualize ER immunostaining intensity and distribution on the paraffin sections. Immunohistochemistry is a technique used to detect proteins in tissue sections with specific antibodies connected to enzyme markers, which form colored precipitates.

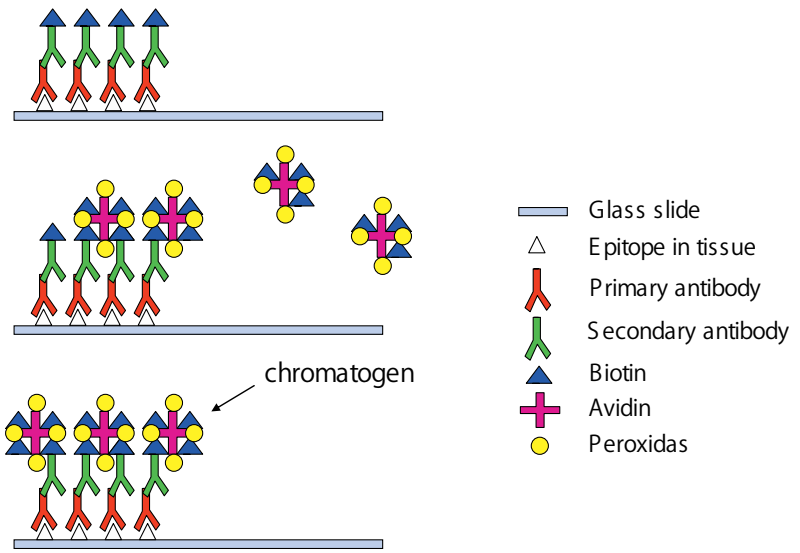

Figure 7. *Schematic explanation of the immunohistochemical technique using avidin-biotin-peroxidase. The chromatogen, 3, 3´-diaminobenzidine (DAB) is applied to the tissue and incubated with avidin-biotin-proxidase complex, which produces a brown insoluble precipitate and permits visualization of the antigen-antibody binding.*

The summary of the antibodies used for staining in rat tissue are presented in Table 2, and Table 3 summarizes those used in mouse tissue. Antigen retrieval was performed by microwaving the sections for 20 minutes in 10mM citrate buffer at pH 6.0. After washing in buffer (0.1M PBS, pH 7.4 for ER, ERα, and ERβ (PA1-310B); and 0.1M Tris-buffered saline (TBS), pH 7.4 for ERβ), non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 10 minutes at room temperature (RT). Following a 10 minute wash in buffer, sections were exposed to a 30 (ER, ERα) or 60 (ERβ) minute non-specific block using diluted normal goat serum, NGS (DAKOCytomation, Carpinteria, CA) in PBS (ERα; ER and ERβ:PA1-310 in Paper II); PBS containing 2% bovine serum albumin, BSA (ERβ:PA1-310 in Paper I); or TBS containing 1% BSA (ERβ) in a humidified chamber at RT.

The tissue sections were thereafter incubated with primary antibody diluted according to the Table (2 and 3) and incubated overnight at 4^{o}C (ERα; ERβ:IgY 503; and

ERβ:PA1-310 in Paper I); for 60 minutes at 4°C (ERβ:PA1-310B in Paper II); and ER which was incubated for 60 minutes in RT. Following primary antibody binding, the sections were incubated for 30 (ERα), 45 (ERβ:PA1-310 in Paper I) or 60 (ER:ZS08-0174 and ERβ:PA1-310 in paper II) minutes at RT with secondary antibody, a biothinylated goat anti rabbit IgG (Vector Laboratories), diluted in NGS, except for ERβ:PA1-310 in Paper II which was diluted in NGS with 2% BSA.

ERβ (IgY 503) was incubated for 60 min at RT with biothinylated goat anti chicken IgY-β (Santa Cruz Biotechnology, CA, USA), diluted in NGS containing 1% BSA. Thereafter the sections were incubated for 30 minutes at RT with horseradish peroxidase-avidin-biotin complex (Vectasain Elite, Vector, CA, USA).

The antigen-antibody binding was detected with the application of 3, 3´-diaminobenzidine (K-3466, DAKO Cytomation, Carpinteria, CA), a chromatogen which produces a brown insoluble precipitate when incubated with enzyme. Thereafter, sections were counterstained with haematoxylin and dehydrated before mounted with Pertex (Histolab, Gothenburg, Sweden). The results of the staining were analyzed with light microscopy.

Tissues from uterus (ERα) and ovaries (ERβ), served as positive controls. These tissues are known to have high expression levels of the studied estrogen hormone receptors. Negative controls were obtained by replacing the primary antibodies with equivalent concentration of rabbit IgG for staining with ERα and ERβ (PA1-310). For staining with ERβ (IgY 503) negative controls were obtained by replacing the primary antibody with non-immune serum of equivalent concentration or by pre-absorbing ERβ (IgY 503) with ERβ protein (Panerva, Madison, WI, USA).

Table 2. *Primary antibodies for detection of rat antigens used in this study.*

Antibody	Directed against	Catalog #	Host	Type	Dilution	Source
ER-α	estrogen rec. α	RM-9101	Rabbit	mono	1:100	Lab Vision
ER-β	estrogen rec. α	PA1-310B	Rabbit	poly	1:500 (Paper I) 1:1000 (Paper II)	ABR
ER	estrogen rec. α and β	ZS08-0174	Rabbit	poly	1:100	Zymed

Lab Vision (Lab Vision Corporation, Fremont, CA USA), ABR (Affinity BioReagents, Golden, CO, USA), Zymed Lab (Zymed Laboratories, South San Francisco, CA, USA)

Table 3. *Primary antibodies for detection of mouse antigens used in this study.*

Antibody	Directed against	Catalog #	Host	Type	Dilution	Source
ER-α	estrogen rec. α	RM-9101	Rabbit	mono	1:100	NeoMarkers
	estrogen rec. α	MC-20: sc-542	Rabbit	poly	1:100	Santa Cruz
ER-β	estrogen rec. β	ERβ: IgY 503	Chicken	poly	1:100	a(Saji et al.,2000)

NeoMarkers (now a part of Lab Vision Corporation, Fremont, CA USA), Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)

a- Kindly provided by Prof. J-Å Gustafsson, Dept. of Biosciences and Nutrition, Karolinska Institutet, NOVUM, 141 86 Huddinge, Sweden

3.5 IMMUNOFLUORESCENCE (DOUBLE) STAINING (III)

In order to visualize both subtypes of ERs at the same time in the same tissue section, a double immunofluorescence staining method was used. Double staining of ERα and ERβ in 12-month-old WT mice was performed as described by Reid et al (Reid et al., 2004). For staining of ERα, a monoclonal rabbit anti-human ERα antibody (RM-9101, Neomarkers, Fremont, CA, USA) was used, and for ERβ, a chicken anti-rat/human antibody (anti-ERβ: IgY 503, (Saji et al., 2000)) was used.
Secondary antibodies were used as follows: for green fluorescence - Alexa 488 (goat anti-rabbit), Invitrogen, A11008, at a dilution of 1:500; for red fluorescence – Cy-5 (donkey anti-chicken), Jackson Laboratories, at a dilution of 1:500.

3.6 IMAGE ANALYSIS (I-III)

To assess the immunostaining photographs, a Leica microscope connected via video camera (Sony) to a computer using Colorvision software (Leica Qwin, Leica Imaging System, Cambridge, UK) was used (Paper I-III). The morphology photographs and the immunostaining images in Paper II were obtained with Zeiss Axioplan microscope with PixeLINK camera.

Three different researchers, independent of each other, classified all inner ear specimens, using a light microscope and a graded scale: -, negative (no) staining; +, positive staining; +*, strong positive staining but in few cells (Paper I), and -, no staining; +, slight staining, and ++, staining (Paper II). In this way, we were able to assess semi-quantitative values from immunohistochemistry.

The immunoflourescence stained sections were visualized with a Zeiss LSM510 confocal microscope, using excitation wavelengths and filters appropriate for the fluorophores (Alexa 488 and Cy-5). Pinhole settings were optimized for the objective lens used. All images were acquired in the sequential scanning mode to minimize bleed-through.

3.7 QUANTIFICATION OF SPIRAL GANGLION CELL DENSITY (III)

The quantification of spiral ganglion cells was performed at the basal and apical turns in the cochlea in WT- and ERβ-/- mice at 3 and 12 moths of age.
A single section of the mouse cochlea prepared for histological analysis contained 4-5 cross-sections of the spiraling Rosenthal's canal (where the spiral ganglion is situated) at locations from the base to the apex. As successive sections were cut, when the mid-modiolar plane was reached (i.e., when all 4-5 cross-sections of Rosenthal's canal were visible in the same section), every section (4 μm thick) was collected, mounted on glass slides and stained with Paragon. Six consecutive mid-modiolar sections were assessed for spiral ganglion neuron (SGN) density. All neurons fulfilling the size and shape criteria to be considered large type I SGN's (i.e., cell diameter 8-20 μm with a nucleus

3-6 μm in diameter) within each profile of Rosenthal's canal were counted. The outline of the Rosenthal's canal profile was traced (using Sigma ScanPro 4 software) to estimate the area. The SGN profile density was then expressed as the number of SGN profiles per 10,000 μm^2. A total of 6 cross-sections of the Rosenthal´s canal were analyzed in each animal. Four animals in each age group were examined.

3.8 STATISTICS (III)

For the statistical analysis in the ABR study (paper III) the Linear Mixed Models was used, which is more modern and flexible approach than the classical method of Repeated Measures Analysis of Variance (ANOVA).
We did not use t-test, in a univariate manner, due to the characteristics of the data.
ANOVA has the shortcomings of having strict assumptions, such as equal variance across groups, equal variance at each repeated measure and equal dependence between pairs of repeated measures. These assumptions can be relaxed by using the more flexible framework of the "Linear Mixed Models". The dependence, due to repeated measures made on the same subject (animal), can be modeled by estimating the covariance/correlation between repeated measures. To avoid data driven results, restrictions on the covariances/correlations are usually performed, and a parsimonious model is estimated. To decide which method to use, several information criteria have been proposed by the statistical literature (e.g. Aikake's Information Criteria (AIC) and Bayesian Information Criteria (BIC)) (Burnham and Anderson, 2002). In the analyses performed in this paper, the corrected Akaike's information criteria (CAIC) was used (Akaike, 1974). A qualified statistician conducted the statistical analyses.

For the calculation of the spiral ganglion neuron density one-way ANOVA, was used. Data are presented as mean ± SEM.

4 SUMMARY OF RESULTS

4.1 EXPRESSION PATTERN OF ESTROGEN RECEPTORS IN THE INNER EAR (I-III)

4.1.1 Estrogen receptors are present in the cochlea of rat and vary during maturation and pregnancy (I)

Estrogen receptors α and β are present in the inner ear in certain stages of the lifecycle and were found to be up- and down-regulated depending on the stage of maturation, development and pregnancy (Table 4). During pregnancy and maturation the estrogen receptor levels in the inner ear vary over time, mirroring the fluctuating estrogen levels in the blood. At the time points measured in non-fetal rats, ERα and ERβ expression was highest in 21-day-old rats (P21, when estrogen levels are known to be low), and reached the lowest level in pregnant mother rats on day 18 of pregnancy (when estrogen levels are known to be high). Seemingly, high levels of estrogen down-regulate the estrogen receptors thus suggesting an active role of estrogen on the hearing organ in the postnatal female rat.

Different from the inverse correlation of estrogen levels and estrogen receptor expression found in non-fetal rats, rats in the fetal stage appeared to have no ER receptors expressed at all. There was no ERα or ERβ identified in the inner ears of the fetuses, at either E8 or E18 (embryological/gestational age of 8 and 18 days respectively).

Table 4. *Expression pattern of ERs in the inner ears of tested rats.*

Status of rat	Spiral ganglion large cells		Spiral ganglion small cells		Spiral ligament		Organ of Corti OHCs		Stria Vascularis		Limbus	
	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ
P21 (21 days)	+	-	-	+	+	+	+	+	+	+	+	+
P56 (56 days)	+ *	(+)	-	-	-	-	-	-	-	-	-	-
Pregnant mother rat d 8	+ *	-	-	+	+	+	-	-	-	-	-	(+)
Pregnant mother rat d 18	+	-	-	-	-	+	-	-	-	-	-	-
Fetus E8 + E18	-	-	-	-	-	-	-	-	-	-	-	-

*P21, 21 day old rats; P56, 56 day-old-rats; E8, fetuses at embryonic/gestational age 8 day and E18, day 18; + positive staining; (+) faint positive staining; - negative (no) staining; + * strong positive staining but in few cells.*

4.1.2 Distribution pattern of the two ER subtypes in the inner ear (III)

WT

In the mouse spiral ganglion, both at 3- and 12 months, large ganglion cells were preferentially stained with ERα and small ganglion cells with ERβ.
The studies on distribution pattern of the two ER subtypes in the inner ear were done on 12 months old WT mice with immunohistochemical and double immunofluorescence technique. ERα was found to be present in large spiral ganglion cells and weak ERα staining in stria vascularis and no staining in Organ of Corti; while ERβ was generally present in more nuclei in the inner ear than ERα. ERβ was especially abundant in cells with smaller nuclei in the spiral ganglion. Also, ERβ was abundant in the limbus, stria vascularis and the spiral ligament, which harbor fibroblasts and fibrocytes. When ERα and ERβ were visualized in the same section, there was some co localization, indicating that the two estrogen receptors are sometimes present in the same cells (see Figure 3 in Paper III).

ERβ-/-

As expected, no ERβ staining was detectible in the inner ear of ERβ-/- mice.

ERα nuclear staining in the inner ear of 3-month-old ERβ-/- mice and WT littermates was indistinguishable from the normal pattern previously described in CBA mice (Stenberg et al., 1999). ERα staining was seen in the large spiral ganglion cells, and in the nuclei of inner hair cells and supporting cells. Weaker staining was found in the outer hair cells. In the stria vascularis the staining of ERα was detected in the marginal and basal cells. Even some nuclei in the spiral ligament were stained.

In 12-month-old ERβ-/- mice in the basal turn of the cochlea, nuclei that stained positive for ERα were seen in the few remaining spiral ganglion cells, but no staining was detected in the Organ of Corti which was degenerated (flat epithelium) or in the stria vascularis. However, in the apical turn of the cochlea, there was ERα staining in the spiral ganglion, weak staining in the stria vascularis, but no staining in the Organ of Corti, except for weak staining in the supporting cells.

4.1.3 Anti-estrogen treatment down-regulates ERα in the stria vascularis (II)

The purpose of the study in Paper II was to investigate the effects of estrogen; estrogen-modulators and anti-estrogen on the ERs in the inner ear in rats. For that reason, the rats were ovariectomized 14 days before initiation of the treatment. When the ovaries are removed, the major estrogen production in the body is halted and not interfering with the outcome of the treatment effect.
The rats were divided in 4 groups. They were treated for 48 h with estradiol, Tamoxifen (mixed agonist/antagonist), ICI182780 (pure antagonist) or vehicle only (propylene glycol).

No difference in the ERs content in the inner ear was observed, except for a possible down-regulation of ERα in the marginal cells of stria vascularis in the group treated with ICI 182780. The control group (injected with vehicle only) showed the same staining as the animals given estradiol or tamoxifen.

4.2 SEVERE HEARING LOSS IN THE ERβ-/- MICE (III)

4.2.1 Hearing threshold

At 3 months of age, there was no statistical difference ($p>0.05$) between ERβ-/- mice and their WT littermates in auditory brainstem responses.
With age, the ERβ-/- mice had a severe hearing loss exceeding that of the WT littermates. By 12 months of age, the hearing was not measurable in the ERβ-/- mice and these mice were considered deaf. As a result the measured values of hearing threshold (dB SPL) did not vary in the 12-month-old ERβ-/-. Also, the WT littermates showed a decline in hearing, but not as severe as the ERβ-/- and the difference in hearing thresholds between the WT and ERβ-/- mice at 12 months was statistically significant ($p<0.05$) for all tested frequencies.

4.2.2 ABR-latencies

When the ABR-latencies were calculated there were no differences, either with regard to the first peak or between peaks I and III, between the two genotypes of 3-month-old mice (ERβ-/- and WT). In WT mice at 3 months of age the latencies were shorter than in mice at 12 months. No latencies could be calculated for the ERβ-/- mice at 12 months, since they were deaf and no ABR waves that were measurable (i.e. the hearing loss exceeded the limit of the equipment).

4.3 INNER EAR PATHOLOGY IN ERβ-/- MICE (III)

Morphology

At 3 months of age, the inner ear morphology of ERβ-/- mice appeared to be indistinguishable from WT mice. The organ of Corti had a normal shape containing inner- and outer hair cells and had an open tunnel of Corti. Hair cells and spiral ganglion cells, both large and small, were present in all cochlear turns.
At 12 months of age, in ERβ-/- mice, in the basal turns, the organ of Corti showed a flat epithelium (Figure 8A). No IHCs and OHCs were observed. There was a major loss of all hair cells in the basal turns, corresponding to an almost total loss of ganglion cells at the same level. Loss of hair cells was also found in the middle and to a lesser extent in the apical turn. In both locations the corresponding ganglion cells were present. There was also loss of hair cells and spiral ganglion cells in the 12-month-old WT mice,

especially in the basal turn of the cochlea, but the loss was not as extensive as that seen in the ERβ-/- mouse.

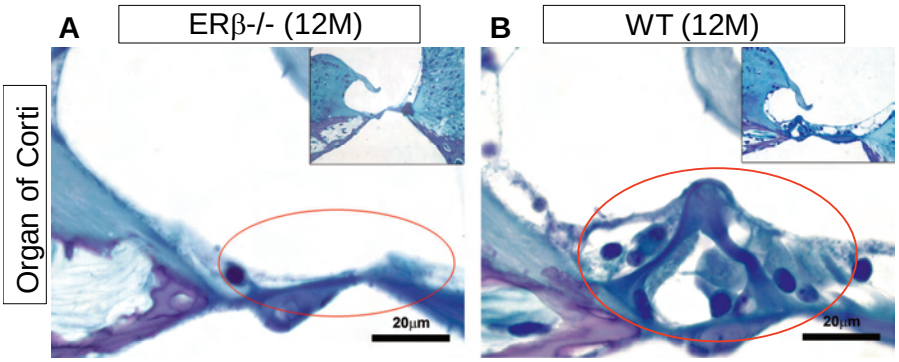

Figure 8. *Morphological photos from the basal turn of the cochlea in 12-month-old mice. Figure A shows a degenerated organ of Corti in a 12-month-old ERβ-/- mouse, resulting in a flat epithelium (red circle). Figure B showing an organ of Corti in 12-month-old WT mouse with better preserved architecture (red circle).*

Quantification of spiral ganglion cell density

The presence of spiral ganglion neurons (SGN) in the base of the cochlea, at 3 and 12 months of age in ERβ-/- and WT mice was measured by calculating the cell density in sections of the Rosenthal's canal at different locations along the length of the cochlea. The SGN profile density (d) is expressed as the number of SGN profiles per 10 000 mm^2.

The density of spiral ganglion neurons in the basal part of the cochlea at 12 months of age was significantly higher in WT mice (d= 16.46 ± 6.34) compared to ERβ-/- mice (d= 6.20 ± 2.93). There was no difference in SGN density in 3 months old mice (ERβ-/- mice: d= 45.12 ± 0.24 and WT mice: d= 44.80 ± 0.24). Both groups had similar high densities in the apex at both 3 and 12 months. At 12 months of age, the mean density, in the base of the cochlea, in the WT mice was about 2.7 times higher than in the ERβ-/- mice.

5 DISCUSSION

In this thesis, a more thorough understanding of sex steroid hormone estrogen, its receptors in the inner ear and effects on the hearing was obtained.
The main findings of this thesis are that ERβ seem to preserve hearing function and that there is a naturally varying expression patterns of estrogen receptors in the inner ear during pregnancy and maturation. These findings are not surprising considering that the female sex hormone estrogen is a hormonal multi-tasker, having roles as diverse as differentiation and function of the reproductive tract, memory storage and bone growth (Petterson and Gustafsson, 2001; Kuiper et al., 1998; Turner et al., 1994).

5.1 DYNAMICAL VARIATION OF ESTROGEN RECEPTORS IN THE INNER EAR

Because the estrogen receptor expression in individual cell types is not a fixed trait, (i.e. receptors may be present or absent in cells under various conditions), it is of interest to study the receptor expression during periods of maximum and minimum hormonal fluctuations. Such hormonally driven conditions, occurring naturally in the body, are among others, fetal development, maturation and pregnancy.

Fetal development

Only human fetuses have previously been investigated for ERs in the cochlea, where only a very week staining of ERα in the spiral ganglion was observed (Stenberg et al., 2001). There are no studies on ERs in rat cochlea during inner ear development. However, in rat brain it has been shown that the ERα expression seems to increase gradually with gestational age, and ERβ was first seen in postpartum pups.

In this study, there was no ERα or ERβ found in any structures of the immature inner ear (i.e. Köllikers organ) in developing rat fetuses, either at gestational age 8 (E8) or 18 (E18). The rat fetus at E8 is thought to be a good model of day 20-post conception in humans, and E18 is equivalent to around day 80. There are differences between rodents and human inner ear in respect of morphology at birth. At birth, in rat, the hearing organ is morphologically mature, but the ear canal is closed. Hearing is not fully developed until pups are 14 days old, when the ear canal opens, and the Organ of Corti is fully mature and innervated in all turns at 21 days post partum (Evans, 2005).
The absence or ERs in the fetus was an unexpected result because low estrogen levels in fetal rats would be expected to cause an up-regulation of estrogen receptors.

Estrogen production is low in fetal tissue, and the vast amounts of maternal estrogen are believed to be neutralized by α-fetoprotein, a fetus specific estrogen binding protein produced in the fetal rat liver (Toran-Allerand, 1984). The lack of estrogen receptors in the fetuses could be due to several factors, among them: a still undeveloped inner ear or

the very low circulating estrogen in the fetal tissue. When examining these fetuses, the sex was not determined, meaning that some of them were presumably males. Regardless of the gender, the fetus is considered to be protected from the vast amount of maternal estrogen secreted from the ovaries by α-fetoprotein.

The absence of ERs in the cochlea of the developing fetus suggests that estrogen does not have an effect on the cochlea during gestation.

Maturation and pregnancy

Of the time points measured in non-fetal rats, ERs expression was highest in 21-day-old rats (P21), and reached the lowest level in pregnant mother rats on day 18 of pregnancy.

In early pregnancy (the first 8 days) in rat, the estrogen concentration in peripheral blood remains low, after which time it significantly increases beginning on day 14 until delivery at day 22, and reaching a maxima on day 21 (Taya et al., 1981). In the current study, early in pregnancy at day 8, the inner ear of the mother rat showed strong ERα staining in few large ganglion cells, while ERβ was found in the small spiral ganglion cells. In the late pregnancy (day 18), there was only positive ERα-staining in the spiral ganglion and spiral ligament, but no ERβ staining. This implies that the high estrogen concentration in the peripheral blood down-regulates the ERs in the inner ear of the postnatal rat, affecting the ERβ expression pattern more strongly than the one of ERα, and suggests that estrogen play an active role on the hearing organ. But the effects of relative expression levels of ERα and ERβ on hearing are still unknown.

Manipulation of the hormone receptors by using estrogen receptor modulating drugs

A popular approach in the field of hormone response is ovariectomy and hormone replacement or antagonist treatment, because this approach allows selective control of the hormonal environment. After being treated with estradiol, Tamoxifen or anti-estrogen (ICI182780), the only changes in ER expression was seen in the group of ovariectomized rats treated with pure anti-estrogen; and the only cells affected by the treatment were the marginal cells of the stria vascularis with a down- regulation of ER. One of the reasons why the stria vascularis was affected and not any other part of the inner ear could be due to its vascularization. Higher levels of vascularization would allow higher delivery of circulating drugs. It is well known that compared to the other areas, the stria vascularis is more vascularized.

Another reason that may explain the low level of changes in the ER expression patterns in the different treatment groups could be the time-period the treatment was given. Perhaps changes in ERs expression pattern in the less vascularized regions need a prolonged time of treatment exposure to occur.

Estrogens, like mineralocorticoids, have certain sodium- and water retaining effects in the renal tubules (Kumagami, 1994); it is also known from earlier studies that the stria vascularis is involved in ion and fluid balance of the endolymph (Ferrary et al., 1996;

Erichsen et al., 2001). The fact that ERα was down-regulated in the marginal cells of stria vascularis after treatment with anti-estrogen, suggests that estrogen have an impact on the ion- and water balance in the cochlea, which could explain the exacerbation of symptoms during the premenstrual phase (low estrogen levels) in women with Ménièr's disease (Andrews et al., 1992), where endolymphatic hydrops is believed to cause the symptoms (Horner, 1991). It has recently been reported by König et al., that megalin, a low density lipoprotein receptor, is present in the same cells (marginal cells) in the stria vascularis as ERβ. The authors also reports that FITC-labeled estrogen is taken up into those same cells. The König study on megalin deficient mice, showed reduced uptake of FITC-labeled estrogen into the strial marginal cells and by the age of 3 months, a profound hearing loss already existed as well as a disturbed expression of potassium channels. Megalin could therefore be a candidate for binding of estrogen or estrogen receptors (König et al. 2008). Based on the findings of König et al., the hearing loss in the ERβ-/- mice could be explained by abnormal water/ion regulation caused by premature degeneration of ERβ–deficient stria vascularis. Also the very weak expression (down-regulation) of ERα in the stria vascularis in ERβ-/- mice could be another factor leading to disturbed water/ion balance with a decrease in the endocochlear potential that could also contribute to the deafness by 12 months of age in these mice. ERα-staining is present in an equal manner in older mice (12 months) of both animal groups, strengthening the hypothesis that ERβ is the main ER isoform protecting against hearing loss.

5.2 INFLUENCE OF THE ERβ ON THE INNER EAR MORPHOLOGY AND AUDITORY CAPACITY

In earlier studies on the brains of estrogen receptor knock-out mice (both ERβ-/- and ERα -/- mice), a significant neural loss and proliferation of astroglial cells was observed in ERβ-/- mice, which corresponded to early brain degeneration; however, these findings where not seen in the brain of ERα-/- mice (Wang et al., 2001).
Because of the close connection of the inner ear with the brain through the auditory pathway one would suspect similar findings in the inner ear of the ERβ-/- mice, which was confirmed in this thesis. The ERβ-/- mice were deaf by one year of age. The observed hearing loss corresponded well to hair cell loss in the cochlea that started in the basal turn and spread towards the apex. The spiral ganglion cells followed the same degeneration pattern, lacking many of its neurons in the basal turn of the cochlea, similar to the one described by Wang et al in the brain of ERβ-/-. These areas all contain cells that normally express ERβ (Stenberg et al., 2002). The findings of this thesis is in concordance with a recent study by Meltser et al. that showed the young ERβ-/- mouse being more vulnerable to acoustic trauma. The ERβ-/- mouse had a greater threshold shift compared to controls, implying a protective effect of ERβ on hearing function (Meltser et al., 2008).

Correlation between the hearing threshold and the inner ear morphology

Although the ABR measurements in 12-month-old ERβ-/- mice were above the equipment limit (no measurable hearing threshold) in all frequencies, even the low

once, we observed a seemingly 'well preserved' morphology in the apical turn of the cochlea of the hearing cells in Organ of Corti and the neuronal cells in the spiral ganglion. With that picture in mind one would expect at least measurable ABR thresholds in the lower frequencies. Obviously, seemingly preserved morphology in the apical turn of the cochlea is not enough for generating measurable hearing in the lower frequency region, and the reason for deafness may be found more centrally.
On the other hand, at same age, the WT mice, showed less altered inner ear morphology than the one seen in ERβ-/- mice in the basal turn. In the apical turn, the morphology status in both groups was similar. Despite that fact, the WT mice had measurable hearing at 12 months, even though they also exhibited hearing loss.

These findings show that there is more than just the morphological condition of the inner ear that matters regarding hearing and suggests that the functional role of ERβ could be one of them. It is remarkable that many genes which, when mutated, cause deafness, are ERβ regulated genes. Connexin 26, cadherin, myosin and procadherin 15 have been found by micro array analysis of prostate, heart, mammary gland and lung to be under-expressed in ERβ-/- mice (Lindberg et al., 2003). Loss of ERβ should, therefore, be expected to be accompanied by loss of or decrease in expression of multiple genes involved in hearing. Mutations in structural genes such as myosin (Donaudy et al., 2003; Kudo et al., 2003), usherin (Pennings et al., 2002; Eudy et al., 1998) and aquaporin (Mhatre et al., 2002) also result in deafness.

Furthermore, both estrogen receptors are present in the inner ear at specific localizations suggesting subtype-specific function: ERα staining was predominant in the nuclei of large neurons and ERβ in nuclei of small neurons and fibroblasts (Paper III). In the spiral ganglion, type I spiral ganglion cells are believed to innervate IHCs, while type II spiral ganglion cells are connected to OHCs (Spondelin, 1972; Spondelin, 1979; Kiang et al., 1984). This indicates that ERα, found mostly in the large (type I) cells, has more effect on the IHCs and sound perception. Consequently, ERβ, staining mostly small cells, including type II spiral ganglion cells, may suggest an effect on the OHCs and the fine tuning of the hearing.

Some spiral ganglion cells stained for both ERs, meaning there is co-localisation of both ERs in the same cell and possibly an interaction between them. Result from Paper I showing variation in ERs expression pattern during different hormonal changes in life implies that the interaction between the receptors and the hormones is highly dependant on the time point of measurements.

In this thesis, only female animals were used. Studies on male cochleae combined with studies on other sex-steroid hormone receptors are needed to give us clarity of the role estrogen and ERs play in gender differences. Also, studies on the effect of aromatase expression, AR and ER expression in both female and male estrogen receptor knock-out mice would give us more knowledge of the role of estrogen as a modulator of auditory encoding in rodents. Our preliminary results on ERα-/- and aromatase knock-out mice show that even these mice have altered inner ear morphology and hearing. In a recent study by Forlano et al., the authors found expression of aromatase mRNA in the ganglion nerve cells of the eighth nerve in a midshipman fish, suggesting that the ear

itself, by converting androgen to estrogen, is a local source of estrogen driven peripheral plasticity (Forlano et al., 2009).

Turner syndrome is a condition where the body is developed in a naturally estrogen deficient environment, due to absent or low estrogen production (Saenger, 1996). It is well known that women with TS develop a progressive sensorineural hearing loss early in life, after age of 35 years (Hultcrantz et al., 1994). In early childhood the girls with TS usually have fairly normal hearing thresholds (Stenberg et al., 1998). When investigating peripheral and central hearing functions in older women with TS the majority of hearing loss was due to peripheral cochlear degeneration. These women also have a more severe and rapid hearing decline than women in the normal population. TS women show a hearing decline comparable to that of 70-year-old women; and a neuro-cognitive dysfunction in sound localization is added to the syndrome (Hederstierna et al., 2008; Hederstierna et al., 2009). Impairment of spatial learning is also present in women with Turner syndrome, and this impairment has also been demonstrated in ERβ-/- mice (Boman et al., 1998; Rissman et al., 2002).
Progressive hearing loss in the high frequency region has also been observed in the "Turner mouse" (missing one X-chromosome) (Hultcrantz et al., 2000). These mice have low estrogen production, but a normal pattern of estrogen receptors (Stenberg et al., 2002), and their hearing loss seems to be of cochlear origin with a central component engaging the eighth cranial nerve, resembling that of the ERβ-/- mice.
In the light of the Turner syndrome studies, the theory of the present study could be applied (i.e. ERβ is essential for maintaining hearing functions).

6 FUTURE PERSPECTIVES

There have been many studies, both on animal and on humans to try to shed light on the area of sex steroid hormone's importance for its target organs and their functionality in the body. Although this thesis is focused on pre-clinical basic research, the goal is to find a suitable treatment regime or preventative strategies for the patients who are suffering or are at risk for developing hearing impairment that is hormonally driven.

Even though studies suggest that estrogen may have beneficial effects on the inner ear and hearing (Kilicdag et al., 2004; Hederstierna et al., 2007), the use of HRT for the explicit purpose of maintaining hearing function or slowing down age-related hearing loss can be a controversial issue and still too early to recommend. New, estrogen receptor agonists are under development and would be beneficial for prevention of age-related hearing loss, in the same time avoiding the estrogens unwanted side effects, such as increased risk of uterine cancer.

6.1 PROGESTERONE AND ANDROGEN RECEPTORS IN THE INNER EAR

Preliminary results of immunohistochemistry mapping of PRs and AR in the rat and human inner ear have failed to show presence of the receptors in the inner ear. So far, only a positive cytoplasmatic staining of PRB in the large spiral ganglion cells was observed in the human cochlea. More experimental studies and use of various antibodies are needed in order to draw conclusions about androgen and progesterone receptors at this time.

6.2 DRUG ADMINISTRATION PATHWAYS

One growing experimental filed is the search for alternative drug administration pathways. Today, the administration of estrogen supplementation is usually oral or by diffusion through the skin, both aiming a higher plasma concentration level and thus increasing the risk of hormonally driven tumors. Regarding the need for estrogen supplementation in preserving hearing function, one possible therapy could be to locally administrate estrogen in the middle ear, through the tympanic membrane, and then by diffusion via the round window in to the inner ear; or by an implantable pump in to the perilymph. This is an exciting field and effort is being put forth to find practical ways to transport the active substance into the inner ear (Saber et al., 2009; Garnham et al., 2005). The positive aspect of local drug administration would be avoiding the systemic side effects estrogen has on the other organs in the body (e.g. the risk for uterine cancer), and also allow more accurate dosage.

6.3 ESTROGEN AND ANTI-ESTROGEN TREATMENT EFFECT ON THE HEARING

Estrogen treatment

Starting in the 80's, young Turner girls/women have been treated with estrogen and growth hormone. They are now approaching 30 years of age and now their hearing will be tested. This presents a unique possibility to compare their audiograms with those who have not been undergoing estrogen supplementation (i.e. the earlier audiograms of now elderly Turner women). More experimental studies regarding Turner syndrome can be performed on a mouse model, the 'Turner mouse', lacking one X chromosome. Although these mice have no estrogen production, they have ERs present in the inner ear (Stenberg et al., 2002). An ongoing study will try to shed some light on the effect of estrogen supplementation on the inner ear and on hearing in Turner mice.

Negative effects of anti-estrogen treatment

A group of women have lately been observed to have negative effects on hearing due to anti-estrogen treatment. These women were receiving anti-estrogen treatment as part of the treatment for breast cancers that are estrogen receptor-positive. The results of this recently initiated study will provide more information of the anti-estrogen treatment effect on the hearing, and will be interesting to compare to a study on men who receive estrogen treatment as a step in therapy for prostate cancer.

All together, the findings of this thesis provide a better understanding of HRT in patients with low estrogen levels (e.g. postmenopausal women, girls with Turner syndrome, and women on anti-estrogen therapy).
Nevertheless, many fundamental questions remain. Further investigation of sex hormone receptors and hearing is merited including elucidation of: the protective role of ERβ on hearing along with its possible interactions with ERα; the complex interactions of the reproductive hormones in the inner ear along with their effects on target organ morphology and hearing, and localization and functionality of other reproductive hormone receptors.

7 CONCLUSIONS

The following conclusions were drawn based on the main findings of this thesis:

- Estrogen receptors α and β are up- and down-regulated in the inner ear depending on the stage of maturation, development and pregnancy. This suggests that estrogen may have an effect on the cochlea during various stages of life. No estrogen receptors were found in the cochlea of the developing fetus, indicating that estrogen does not have an effect on the cochlea during gestation.

- Treatment of ovariectomized rats with estradiol, tamoxifen, anti-estrogen or vehicle only, does not alter the ER expression pattern significantly in the inner ear. However, a slight down regulation of ERα in the marginal cells of the stria vascularis (involved in ion regulation) was seen in the rats that where injected with anti-estrogen, suggesting that ERα, and therefore estrogen, may be involved in the ion regulation in the cochlea.

- Both estrogen receptors are present in the inner ear at specific localizations indicating subtype-specific functions. ERα staining was found to be predominant in the nuclei of large neurons and ERβ in nuclei of small neurons and fibroblasts.

- Mice deficient of ERβ, have altered inner ear morphology and are deaf already by one year of age. These findings strengthen the hypothesis that estrogen has a direct effect on hearing and may imply that ERβ is important for the prevention of age-related hearing loss.

8 ACKNOWLEDGEMENTS

To Professor **Malou Hultcrantz**, MD PhD, my supervisor. Thank you for making all this possible! For introducing me to the world of science. For always being there and believing in me. Your creativity and enthusiasm guided me into many thrilling areas and helped me through the rough times…and there were many of them! I sincerely appreciate and admire your extraordinary persistence, enthusiastic personality and willingness to always find time for me whenever I called on you.

To **Annika Elmqvist Stenberg** MD PhD, my co-supervisor, colleague and friend, for your optimism and support. For your help with writing the articles, "taking" our research to the archipelago, and showing me how to mix "business with pleasure". It is a great privilege to have you as a co-supervisor!

To Professor **Dan Bagger Sjäbäck**, MD PhD, and Professor **Eva Munck-Wikland**, MD PhD, for your support and encouragement.

To Associate Professor **Richard Kuylenstierna**, MD PhD, Head of the Department of Otorhinolaryngology, for your support and for creating a unique atmosphere at the department that fosters a collaborative research environment and for giving me the opportunity to complete this thesis.

To my former clinical supervisors: Professor **Göran Laurell**, MD PhD and **Bertil Lundberg**, MD, for your support and for professional and friendly advice whenever needed.

To my co-authors: Associate Professor **Lena Sahlin**, PhD, for letting me be a part of your lab and extensive contributions to the manuscripts; **Britt Masironi**, for sharing your vast knowledge about immunohistochemistry, for all your technical assistance, for all your invaluable support and for being willing to contribute your outstanding problem-solving skills to this project; Professor **Jan-Åke Gustafsson**, MD PhD, for good scientific discussions and successful collaboration; **Anders Fridberger**, PhD, for the help with the confocal microscopy; and **Maoli Duan**, MD PhD, for the help with the ABR-measurments.

To all the people at the G-5 Lab, for creating a nice atmosphere and helping me with various things. I wish to especially thank **Anette Fransson** and **Paula Mannström**, for helping me at the lab, teaching me all about embedding and sectioning and making my life less stressed.

To **Natalie Wisniewski**, PhD, my very good friend and the greatest Editor one can possibly wish for. Thanks for the outstanding linguistic help and for our endless scientific and relationship discussions. You are becoming an expert on the inner ear research now!

To **Mikael Eriksson**, for always making time to help me with the microscopy images.

To **Caroline Gahm**, MD PhD, my friend and colleague, for our discussions about science, and for sharing your knowledge about antibodies, immunofluorescence staining and life itself.

To **Cecilia Engmér Berglin**, MD, colleague and traveling research partner, for making research more enjoyable and glamorous.

To all my wonderful colleagues at the ENT-clinic, for an inspiring and creative atmosphere and for your friendly support.

To my office-mates: **Linda Marklund**, MD PhD, **Elin Marsk**, MD, **Victoria Hellberg**, MD, **Claudia Priwin**, MD PhD, and most of the time even **Lalle Hammarstedt**, MD PhD; for keeping high spirits and for keeping me going with copious amounts of candies as I wrote this thesis. To **Sushma Nordemar**, MD PhD, for your friendship and for being my workout companion. I promise to be a more frequent visitor at the gym now that I am done with this book.

To all my dear friends outside my clinic, particularly: **Neda Rajamand Ekberg**, **Åsa Fröberg**, **Johanna Ungerstedt**, **Sofie Paues Göranson** and **Malin Vading** for your enthusiastic support and the joy you bring in my life.

To **Nazanin**, my friend and "party"-partner, for making my life outside the Karolinska building more glamorous.

To my family: my **parents** and especially my brother **Boris**; my **aunt** and **uncle** in Macedonia. Thank you all for your unconditional love and for always supporting and believing in me!

To **Johan**, for being in my life.

To all mice and rats that gave their lives to make this thesis possible!

To the organizations that provided the funds to make this work possible. The research conducted for this thesis was supported by grants from The Acta Otolaryngologica, The Foundation for Audiological Research (Stingerfonden), The Swedish Research Council (VR), The Karolinska Institutet Funds, The Karolinska Institutet Travel Grants, and The Hirsch Fellowship.

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