Importance of estrogen receptors in early life stages of zebrafish (Danio rerio) development

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Importance of Estrogen Receptors in early life stages of Zebrafish (*Danio rerio*): Development

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ETH ZURICH

For the degree of Doctor of Sciences
Presented by

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SUMMARY

In vertebrates, estrogens exert a variety of diversified effects in many different target tissues and are known to have multiple functions in reproduction, especially in the development of the gonads in female and male. Moreover, a function in non-reproductive tissue, such as a role in neuronal growth and differentiation in the central nervous system (CNS) has been reported. Estrogens act on target genes mainly via the genomic pathway through binding to their nuclear receptors, called estrogen receptors (ERs). In zebrafish (*Danio rerio*), three subtypes have been characterized, ERα, ERβ₁ and ERβ₂. In addition to natural ligands, synthetic estrogens (xenoestrogens) are able to bind to these receptors, thereby activating or inactivating a number of genes leading to altered production of proteins in estrogen target cells. These compounds, such as the pharmaceutical estrogen, ethinylestradiol (EE₂), are known to be released into the environment and can affect the endocrine system of aquatic organisms.

The objective of this thesis is to investigate whether ERs play a role in early developmental processes in zebrafish. These novel roles would enrich our knowledge on non-reproductive functions of estrogens, and thus help to identify new targets for xenoestrogens in embryonic development.

As a first step toward elucidating ERs’ importance, the relative transcript abundance of the three subtypes was measured during normogenesis. All the three subtypes were present since the beginning of embryo development. ERβ₂ subtype was dominant, as the highest level of mRNA was measured. A clear increase of transcript level of ERα was measured upon exposure to EE₂; on the contrary, the expression of ERβ₁ and ERβ₂ mRNA was slightly reduced or not affected. These results suggested that ERβ₂ might be the main mediator of natural action of estrogens, and that ERα might be the subtype responsible for endocrine disruption in early life stages.
The study on the localization of the three subtypes revealed the presence of ER\(\beta_2\) in the head and in the neuromasts, the organs of the lateral line. The role of this subtype was further analysed during embryogenesis by blocking the ER\(\beta_2\) protein synthesis with morpholinos. The ER\(\beta_2\) morphants showed abnormal neuromast development and swimming behaviour. They swam in circles. The effects of the knock down on the neuromasts were analysed in more details. Neuromasts are composed of sensory hair cells and supporting cells. A normal development of supporting cells, but a loss of hair cells has been revealed, suggesting a function of ER\(\beta_2\) in hair cell differentiation.

When searching for molecular mechanisms affected by the ER\(\beta_2\) knock down, an activation of the Notch signalling pathway was observed. This is an essential pathway for guiding cell fate decisions during development and in particular during neuromast maturation. In morphants, members of the Notch family were up-regulated. Consequently, the supporting cells of the embryos missing ER\(\beta_2\) did not differentiate into hair cells, which explains their absence. These findings suggest, therefore, a cross-talk between estrogens and Notch signalling pathways.

In the last part of this thesis, the feasibility of using neuromasts in ecotoxicology for assessing subtle effects of toxicants is discussed. This new approach was evaluated by reviewing the different studies analysing the mechanisms of the process of neuromast development and those assessing the chemical impacts on these organs. The great understanding of neuromasts development enables the use of these organs for analysing toxicological modes of action of different chemical compounds.

In conclusion, this thesis revealed a non-reproductive function of ERs in early development, namely a role during lateral line organs development. These results increased the diversity of estrogen-dependent processes and gave new insights in endocrine disruption research. Moreover, neuromast appeared to be an excellent endpoint for future risk assessment.
RÉSUMÉ

Chez les vertébrés, les estrogènes exercent des effets diversifiés dans de nombreux tissus cibles et sont impliquées dans de multiples fonctions durant la reproduction, comme par exemple le développement des gonades chez la femelle et le mâle. En outre, en dehors des fonctions liées à la reproduction, les estrogènes jouent un rôle dans la croissance neuronale et la différentiation du système nerveux central par exemple. L’action hormonale est principalement véhiculée par le chemin génomique, c’est-à-dire par la liaison de l’hormone avec des récepteurs spécifiques, nommés récepteurs estrogéniques (ERs). Dans le poisson zèbre (*Danio rerio*), trois types de récepteur ont été caractérisés, ERα, ERβ₁ et ERβ₂. En dehors du ligand naturel, des estrogènes synthétiques (xéno-estrogènes) sont capables de se lier à ces mêmes récepteurs, activant ou inactivant ainsi de nombreux gènes, ce qui résulte en une altération de la synthèse de protéines dans les cellules cibles des estrogènes. Par conséquent ces substances, comme par exemple l’estrogènes pharmacétique, ethinylestradiol (EE₂), présents dans l’environnement, peuvent affecter le système endocrinien des organismes aquatiques, processus appelé perturbation endocrinienne.

L’objectif de cette thèse est de découvrir si les ERs jouent un rôle durant l’embryogénèse du poisson zèbre. Ceci permettra d’enrichir notre connaissance sur les fonctions des estrogènes non liées à la reproduction. Sur la base de ces résultats, de nouvelles cibles pour les perturbateurs endocriniens peuvent être identifiées durant le développement embryonnaire.

Premièrement pour élucider l’importance des ERs, la quantité relative de transcrits des trois types de récepteurs a été mesurée durant la normogénèse. Les résultats indiquent que les trois ERs sont présents depuis le début de l’embryogénèse, et que le récepteur ERβ₂ est dominant, puisque la plus grande quantité d’ARNm a été mesurée. Une nette augmentation du niveau de transcrits du récepteur α a été observé suivant l’exposition à
EE₂ ; au contraire l’expression de l’ARNm des récepteurs β₁ et β₂ est légèremment réprimée ou inaffectée. Ces résultats suggèrent que ERβ₂ serait le principal médiateur de l’action des estrogènes naturelles et que ERα serait le responsable de effets de la perturbation endocrinienne durant l’embryogénèse.

L’étude sur la localisation des trois récepteurs dans l’embryon révèle la présence de ERβ₂ dans le cerveau et les organes sensoriels de la ligne latérale, appelés neuromastes. Le rôle de ce récepteur est ensuite analysé durant l’embryogenèse en bloquant la synthèse de la protéine ERβ₂. Le phénotype résultant fut un développement anormal des neuromastes et une anomalie du comportement natatoire. Les embryons injectés avec le ERβ₂ MO (morphants) nagèrent en cercle. Les effets du knock down sur ces neuromastes, composés de cellule sensorielles ciliées et de cellules de support, ont été analysés en détails. Un développement normal des cellules de support, mais une absence de cellules ciliées a été découvert, suggérant une fonction de ERβ₂ durant la differentiation des cellules ciliées des neuromasts.

En recherchant les mécanismes moléculaires affectés dans le knock down de ERβ₂, une activation des voies de transmission du signal par les récepteurs Notch a été observée. Ces voies de transmission sont essentielles pour la détermination du destin des cellules durant le développement et particulièrement durant la maturation des neuromastes. Chez les morphants, des membres de la famille des récepteurs Notch sont induits. En conséquence, les cellules de support des morphants ne se différencient pas en cellules ciliées, d’où l’absence de ceux-ci. Ces résultats suggèrent ainsi un cross talk entre la voie de transmission du signal des estrogènes et des récepteurs Notch.

Dans la dernière partie de ce travail, la possibilité d’utilisation des neuromastes en écotoxicologie est proposée pour évaluer les effets subtils des composés toxiques. Cette approche novatrice a été démontrée en résumant les différentes études analysant les mécanismes de développement de ce processus et celles évaluant l’impact des produits chimiques sur ces organes. La connaissance détaillée du développement des neuromasts permet l’usage de ces organes pour l’analyse du mode d’action toxicologique de certains composés chimiques.

Pour conclure, cette étude a révélé une fonction non reproductive des ERs dans le développement embryonnaire, c’est-à-dire un rôle durant le développement des organes de la ligne latérale. Ces résultats ont accentué la diversité des processus dépendants des estrogènes et a permis des aperçus novateurs dans l’étude des perturbations endocriniennes. De plus les neuromastes se sont avérés d’excellent organes cibles pour de futures analyses de risque.
CHAPTER 1

INTRODUCTION

1.1 Framework

Estrogens and their corresponding receptors might be one of the most studied signalling pathways, which attracted a lot of interest in many different fields of biology, such as endocrinology, toxicology and bioinformatics. The estrogen receptor (ER) has been first identified in rats by Elwood V. Jensen at the University of Chicago in the 1950s. Nowadays, after more than fifty years of research, unexpected results are still published every year. This thesis, studying ERs’ importance in early life stages of zebrafish, delivered interesting and surprising results as well. In the next sections, the main background information and the objectives of this thesis are introduced.

1.1.1 Estrogen Receptors in Vertebrates

In vertebrates, as well as in some invertebrates, estrogens are known to have multiple functions in reproduction, especially in the development of the gonads in female and male. Estrogens are also known to play a role in the brain, regulating sexual differentiation and behaviour. The molecular action of estrogens is mediated through two distinct signalling pathways, the non-genomic (beyond the scope of this thesis) and the genomic pathway, where estrogens bind to their corresponding receptors (Figure 1.1). The ERs are members of the nuclear steroid/thyroid hormone receptor superfamily and share a common structure-function organization (Duffy, 2006; Nilsson et al., 2001; Gruber et al., 2002). There are two ER subtypes in mammals, ERα and ERβ, localized on different
1.1 Framework

chromosomes (Meltser et al., 2008). Being the product of two independent genes suggests an existence of distinct biological roles of each subtype (Gustafsson, 1999).

![Figure 1.1: Mechanisms of nuclear receptor action. From Zilliacus, J. 2007. Hand-out from the course in receptor-mediated toxicity, Stockholm, Sweden.](image)

The mechanism of action of ERs

The natural ER ligands are the endogenous estrogens, 17β-estradiol (E2) and estrone. Being lipophilic molecules, estrogens can diffuse passively across cell membranes and bind to the ERs, located in the nucleus (Kimbrel and McDonnell, 2003). When estrogens bind to ER, the receptor undergoes a conformational change that facilitates dimerization and subsequent interactions with the specific DNA sequences, called estrogen responsive elements (EREs), which are located within the promoter of a target gene and regulate transcription of this gene. The ERE is a DNA motif with palindromic structure that has been identified in estrogen target genes such as vitellogenin and aromatase (Hyder et al., 1995). The receptor comprises a zinc-finger motif DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD), which is composed of 12 α-helical structures (Figure 1.2). The DBD shows a high degree of homology between ER subtypes (only three amino acids differ) and the LBD exhibits only 59% homology, which may explain the different binding affinities for some ligands (Gustafsson, 1999). The large hydrophobic cavity of the LBD allows also the binding of a wide variety of non steroidal compounds through hydrophobic interaction.

ERs interact with several co-regulatory proteins at their two activation sites, activator protein-1 (AP-1) and AP-2 within the LBD. Regulatory proteins intercalate between the activated receptor and the transcriptional machinery. ERs, together with their tran-
scription factors, coactivator or corepressor proteins provide stability and transcriptional specificity in the cells (Gruber et al., 2002).

Some of ER ligands act as agonists or antagonists. The agonistic compounds enter into the ligand binding pocket and activate a conformational change. This enables the helix 12 to take the right position, allowing the coactivator to bind within the pocket and thereby activate transcription. When an antagonist is bound, a conformational difference in the receptor causes helix 12 to block the coactivator pocket, inhibiting correct activation of transcription (Kimbrel and McDonnell, 2003).

The way by which ligands control the receptor activity is much more complex than the conversion from an "off" to an "on" state after binding. It depends on both ERE-promoter context and on ER subtype. In fact, selective estrogen receptor modulators (SERMs), which are non-steroidal ER ligands such as tamoxifen, showed that some compounds can display tissue-specific agonist/antagonist activities (Gruber et al., 2002; Watanabe et al., 1997). Therefore, some ligands acting through the same receptor can manifest different activities in different cells.

Also, a heterodimerization between ERα and ERβ is possible when the two subtypes are coexpressed. That could explain how ERβ can dampen ERα’s transcriptional activity in response to physiological levels of estradiol (Hall et al., 2001; Kuiper et al., 1998b; Lindberg et al., 2003; Maruyama et al., 2001). In fact, in a number of different cell types, for example in the bone of mice, ERβ modulated ERα-mediated gene transcription supporting a "Ying Yang" relationship between ERα and ERβ (Lindberg et al., 2003).

Figure 1.2: Domain structure of nuclear receptor. From Ziliacus, J. 2007. Hand-out from the course in receptor-mediated Toxicity, Stockholm, Sweden.
1.1 Framework

ERs distribution and function

Beside the reproductive function of estrogens, different studies analysing the localization of the ERs and the estrogen producer enzyme, the aromatase, revealed an expression in non-reproductive tissues as well. The specific role of each ER subtype in the different tissues has been indicated by the specific phenotypes of ERα and ERβ knock out (ko) mice. Non-reproductive functions of estrogens that have been discovered so far include the regulation of adult bone metabolism, the development of the cardiovascular system as well as neuronal growth and differentiation in the central nervous system (CNS). In the next paragraph the distribution and roles of ERs in mammals and fish will be discussed.

ERs in mammals

The ER subtypes α and β are differentially expressed in mammals. Both are expressed in the reproductive and non-reproductive adult rat tissue. ERα mRNA is predominantly expressed in the uterus, mammary gland, testis, pituitary, kidney, liver, heart and skeletal muscles (Walker and Korach, 2004; Shughrue et al., 1997, 1998). ERβ mRNA is predominant in the ovary, prostate, lung, bladder, gastrointestinal tract, salivary gland, and developing pituitary (Kuiper et al., 1997; Walker and Korach, 2004). An overlapping expression has been detected in the brain, ovary, testis and uterus (Kuiper et al., 1998b; McEwen and Alves, 1999; Walker and Korach, 2004), however, within these tissues that coexpress both receptors, the cellular distribution between the ER subtypes differs (Walker and Korach, 2004).

In the brain ERα is highly expressed in the regions associated with reproductive behaviour such as the hypothalamus (McEwen and Alves, 1999). Weak expression is detected also in hippocampus (Walker and Korach, 2004). These findings are supported by the ERαko mice phenotype. They are infertile and sexual behaviour is absent or greatly disrupted in both sexes (Bodo and Rissman, 2006). These results are in agreement with the estrogen reproductive role in brain development.

ERβ in the brain showed a predominant extra-hypothalamic expression mainly in the hippocampus, cerebellum, cerebral cortex and olfactory bulbs (Gustafsson, 1999). ERβ expression has also been detected in neuropeptidergic subpopulations of neurons known to be important player in the interaction between the endocrine and nervous systems (Bodo and Rissman, 2006). This suggested that estrogens actions mediated by ERβ are related to the various effects on the nervous system that are beyond the hormonal-hypothalamic actions of the regulation of reproductive function. These assumptions were confirmed by studying the brain of ERβko mice embryos. It was smaller with fewer neurons in the
cortex and showed a degeneration of neuronal cell bodies throughout the brain (Wang et al., 2001). Moreover, ERβKO adult mice are reproductively competent, although a reduction of fertility has been described in females, and exhibit normal reproductive behaviour (Bodo and Rissman, 2006). ERβKO mice showed also to have alteration in the learning ability as well as increased anxiety and depression (Bodo and Rissman, 2006). ERβ is therefore necessary for neuronal survival and could have an important influence on the development of degenerative diseases of the CNS. Thus, ERβ showed to be not directly responsible for the regulation of reproductive physiology in rodents, but may mediate the non-reproductive effects of estrogens (Bodo and Rissman, 2006; Gustafsson, 1999).

ERβ is also localized in the inner and outer ear hair cells, as well as in the spiral ganglion neurons in mice (Meltser et al., 2008). These cells are important transducers of acoustic signals to the CNS. In addition, the ERβKO mice and the aromatase KO mice showed to suffer from hearing loss after an acoustic challenge (Hultcrantz et al., 2006; Meltser et al., 2008).

Beside neurons, ERα and ERβ are also expressed in glial cells of the CNS (Gustafsson, 1999; Platania et al., 2003; Mhyre and Dorsa, 2006; Garcia-Segura et al., 1999). It has been shown that astrocytes as well as oligodendrocytes of the rat spinal cord express ERs (Platania et al., 2003; Garcia-Segura et al., 1999). Also, an ER-dependent regulation of neural stem cell (NSC) proliferation and differentiation has been observed (Brannvall et al., 2002). ERα and ERβ are present in rat NSC during early development and in adults with higher levels of ERβ.

In the peripheral nervous system (PNS), little is known about ERs presence. Interestingly, similar to the glial cells in the CNS, the schwann cells are able to synthesize estrogens (Schumacher and Baulieu, 1995) and the ERs antagonist tamoxifen reduced the incidence of schwann cell tumours (Jay et al., 1986). In primary cultures of schwann cells from newborn rat, a presence of different steroid receptors, including ERs, was found. (Jung-Testas and Baulieu, 1998). Thus, the existence of ER in schwann cell are possible. In addition, there are reports on ERα- and ERβ-positive neurons in dorsal root ganglia (DRG), containing cell bodies of sensory neurons, in adult rat and also in cultures (Papka and Storey-Workley, 2002; Patrone et al., 1999). A function of ERs in the development and survival of DRG neurons has been shown, suggesting a possible involvement of ERs in the PNS (Patrone et al., 1999). This role has to be further investigated, and this thesis is contributing to discover further insights in ERs role and presence in the PNS.
1.1 Framework

ERs in fish

In fish, many studies helped to decipher the distribution of the ERs and to understand the pleiotropic effects of estrogens in the body. The ERs subtype story is more complex as in teleost fish many genes are duplicated. For example, the Atlantic croaker (*Micropogonias undulatus*) (Hawkins et al., 2000), largemouth bass (*Micropterus salmoides*) (Sabo-Attwood et al., 2004), sea bream (*Sparus auratus*) (Pinto et al., 2006), fathead minnow (*Pimephales promelas*) (Filby and Tyler, 2005), goldfish (*Carassius auratus*) (Tchoudakova et al., 1999; Ma et al., 2000) and zebrafish (*Danio rerio*) (Menuet et al., 2002; Legler et al., 2002) have in common the particularity to express two ER\(\beta\) proteins with structural differences present throughout their entire coding region, indicating the existence of two distinct ER\(\beta\) forms originating from different genes in these species. The two ER\(\beta\) subtypes share a higher degree of amino acid homology with each other than they do with ER\(\alpha\). They are believed to have arisen from the duplication of an ancestral ER\(\beta\) gene early in the teleost lineage (Hawkins and Thomas, 2004). The only species that showed a duplication of ER\(\alpha\) is the rainbow trout (*Oncorhynchus mykiss*) (Nagler et al., 2007).

This gene duplication complicated the understanding and comparison with other vertebrate studies. A standardized nomenclature has been introduced (Hawkins and Thomas, 2004) but poorly respected even in recent publications (Costache et al., 2005; Leanos-Castaneda and Van Der Kraak, 2007; Nagler et al., 2007), for this reason the old nomenclature has been kept in this thesis. The mammalian ER\(\alpha\) is named ER\(\alpha\) in Atlantic croaker and ESR1 in zebrafish and fathead minnow (ER\(\alpha\) in this study); the mammalian ER\(\beta\) is changed to ER\(\beta\)b in Atlantic croaker and ESR2b in zebrafish and fathead minnow (ER\(\beta_1\) in this study); ER\(\gamma\) identified in the Atlantic croaker became ER\(\beta\)a and ESR2a in zebrafish and fathead minnow (ER\(\beta_2\) in this study) and has not been identified in mammals.

In zebrafish, ERs mRNAs are detected in reproductive and non-reproductive tissues, including the gonads, brain, pituitary, liver, intestine, and eyes (Menuet et al., 2002). The high expression of ERs in gonads has been found in many fish species. In the Atlantic croaker, ER\(\beta_2\) transcripts revealed to be expressed in high levels in the testis.

In the brain of zebrafish, the cellular localization of ERs mRNA exhibited differential but partially overlapping expression pattern in the regions like preoptic tectum, mediobasal hypothalamus and posterior tuberculum, shown to mediate neuroendocrine functions. In the Atlantic croaker ER mRNAs also showed a distinct hypothalamic distribution. ER\(\alpha\) can be detected in the preoptic area, ER\(\beta_1\) is expressed in cells close to the third ventricle and ER\(\beta_2\) in the suprachiasmatic nucleus. The differentiated distribution patterns suggest that each ER gene product may serve distinct neuroendocrine roles.
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A study in fathead minnow revealed an expression of all three receptors during development. A highest expression of ERβ2 transcripts was detected between 5 and 20 days post fertilization (dpf), followed by ERβ1 and ERα mRNA (Filby and Tyler, 2005). High levels of ERα and ERβ1 has been found in the liver, the site of synthesis of the yolk protein precursor, vitellogenin (vtg) (Filby and Tyler, 2005). This gene is known to be stimulated by E2 through the hepatic ERβ in rainbow trout (Flouriot et al., 1997; Leanos-Castaneda and Van Der Kraak, 2007). In the liver of fathead minnow two receptors, ERα and ERβ1, were present in high amount, therefore the E2-dependent transcriptional regulation of the vtg gene may be associated with the coordinated role of these two ER subtypes. However, ERα is thought to be the main mediator of E2 effects in promoting vitellogenesis, as ERα expression increases progressively with sexual development in females. In addition, in zebrafish and largemouth bass a correlation between the up-regulation of ERα and vtg genes by E2 in the liver has been observed. These observations designate ERα as main mediator of this effect (Sabo-Attwood et al., 2004; Menuet et al., 2004).

Early expression of ERs has also been studied in zebrafish embryos/larvae (Tingaud-Sequeira et al., 2004; Bardet et al., 2002; Lassiter et al., 2002). These studies revealed a high abundance of ERβ2 transcript already at 3 hours post fertilization (hpf), as well as ERβ1 expression, suggesting the presence of transcripts of maternal origin. ERα transcripts were only weakly present and were not detected before 12 hpf, but showed a dramatic enhancement around 72 hpf (Bardet et al., 2002). This was also true, but to a lesser extent, for ERβ1 and ERβ2. The study of overall distribution patterns in the embryos showed that ERβ1, ERβ2 and low levels of ERα mRNA can be detected in the epidermis, pectoral fin buds, hatching gland and to a lesser extent in the developing brain. ERβ1 and ERβ2 transcript were found to be expressed in the lateral line organs (Tingaud-Sequeira et al., 2004). These organs will be described in more detail in the section 1.2.2 of this introduction.

1.1.2 Environmental concern

Both scientific community and public are increasingly concerned about synthetic estrogens (xenoestrogens) that, in addition to the natural ligands, are able to activate ERs. Due to this ability, these chemicals showed adverse effects in humans and wildlife leading to endocrine disruption. However, estrogen mimicking is only one of the many mechanisms of action of endocrine disrupting compounds (EDCs). These compounds can also affect steroid synthesis, distribution and excretion. Besides the effects of these chemicals related to androgen and estrogen homeostasis, EDCs can also disrupt the thyroid home-
1.2 Scope of the thesis

Most of current research on ERs’ role in vertebrates is focusing on reproductive functions in gonads or brain. In contrast, this thesis has the ambition to increase our knowledge on
the non-reproductive functions of ERs in early development of zebrafish. In order to fulfil this objective, the zebrafish has been chosen as vertebrate model organism and a specific attention has been given to the role of ERs in the PNS. The background information on the zebrafish and its PNS will be introduced in the following sections.

1.2.1 Zebrafish, a perfect model organism

Zebrafish are part of the Cyprinidae family belonging to the division of teleost fish, which represent a large proportion of all fish. The zebrafish come close to being the ideal model organism for studies on vertebrate development because it combines the best features of all the other models. In addition, increased ethical concern forces investigators to reduce experiments with mammalian models, which are also very expensive to maintain in comparison with non-mammalian vertebrate experimental models. Zebrafish are easy to keep and tolerate harsh conditions, for these reasons being often used as pets in private aquariums. Thanks to their small size, it is possible to breed many individuals in a relatively small room, making the maintenance less expensive. A lot of eggs can be produced every day and offspring reaches sexual maturity at about 3 months. This rapid generation cycle makes zebrafish attractive for many fields in biology. For instance, the developmental biology benefits from the completely transparent embryo, making it possible to observe cell division and developmental processes in a rapid external development (Kimmel et al., 1995). A large scale mutagenesis screen has also been performed, the so called Boston/Tübingen screen, which enabled scientists to discover new genes involved in defects in particular biological processes, such as the developing nervous system. More than 500 mutants were identified in various aspects of early development and are used up to date to address many issues like organogenesis or complex diseases (Haffter and Nusslein-Volhard, 1996; Driever et al., 1996). Many of the zebrafish mutant phenotypes identified in genetic screens are reminiscent of human disease states, providing a powerful approach for identifying corresponding drugs, like it has been shown for Alzheimer’s disease and cancer (Dooley and Zon, 2000; Amatruda et al., 2002). This is mainly possible due to the extensive similarity between the zebrafish and human genome. In fact, many human developmental and disease genes have counterparts in the zebrafish. The zebrafish genome has been fully sequenced and rich information sources, ranging from gene expression to gene function analysis, are available at www.zfin.com.
1.2 Scope of the thesis

1.2.2 Nervous system of zebrafish embryo

Zebrafish is also extensively used in neurobiology. The early brain development in zebrafish embryos is, however, only partially understood. By 5 dpf, all the major components of the brain are present and at this stage there are already well-developed olfactory bulbs (Nüsslein-Volhard and Dahm, 2002). The first neurons are born during gastrulation, soon after the specification of the neuroectoderm at the dorsal side of the embryo. Zebrafish nervous system becomes functional within days, enabling larvae to escape predators and detect food. By two days of age, embryos have a stereotypic escape behaviour, which entails a rapid bend of the body axis away from the source of the tactile stimulus followed by swimming movements. The relative simplicity of the neuronal system of zebrafish larvae makes it a powerful model for studying the development and function of the nervous system (Strahle and Korzh, 2004).

Unlike higher vertebrates, teleosts and amphibians form a primary nervous system consisting of early-born neurons. More neurons are added to the nervous system in subsequent stages, which are referred to as secondary neurons. Recent findings suggested many mechanistic similarities between these two systems and the reason for an early developing, primitive nervous system, may be a survival strategy for free-swimming vertebrate larvae.

The organization of the primary nervous system is pre-figured on the neural plate (Strahle and Korzh, 2004). The neuronal marker Neurogenin1 (Ngn1), a basic-helix-loop-helix (bHLH) transcription factor, and the signalling molecule deltaA are expressed in all areas of primary neurogenesis in the neural plate. Other genes such as the bHLH transcription factor NeuroD appear slightly later in development. Their expression is confined to cells that have already initiated neuronal differentiation and have been selected from the ngn1-positive precursor pool by lateral inhibition. This selection process prevents cells from immediate entry into the neuronal differentiation program. Indeed, initially all cells of the neural plate express ngn1 and have the potential to become neurons (Strahle and Korzh, 2004). However, lateral inhibition mechanisms, which involve the transmembrane receptor Notch and its membrane-bound ligand Delta, inhibit this process. A few cells start to express higher levels of deltaA or deltaD, causing increased activation of Notch in the surrounding cells. This leads to the suppression of ngn1 and delta expression and prevents neuronal differentiation. In other words, Ngn1 expression delineates the areas of neural precursors, some of which express high levels of Delta. Notch is then activated in neighbouring cells leading to expression of repressor proteins that suppress the expression of proneural genes.

The larval development of the PNS is conducted by the DRG neurons which replace the cells of the primary sensory system. The cranial ganglia expand and a well-developed
olfactory and lateral line systems emerge (Hendricks and Jesuthasan, 2004). By 72 hpf most of the basic components of the PNS are distinguishable. Among the quickly developing sense organs are the inner ear, which is required for balance, and the lateral line organ, which detects movement in the water (Hendricks and Jesuthasan, 2004). Both of these sense organs are dependent on mechanosonduction, which is the conversion of mechanical signals into biochemical signals, in sensory hair cells. In this study, the lateral line system received a particular attention, as the most abundant ER is expressed in the lateral line organs, the neuromasts. In chapter 3 more details on the developmental process of these organs are discussed.

1.2.3 Thesis objectives

The goal of this thesis is to increase our knowledge on ERs in early development of zebrafish. To fulfil the gap existing in the embryonic role and expression of ERs, the following research issues were investigated.

In the study described in chapter 2, the aim was to find out how much, in terms of relative abundance, of each ER subtype transcripts are expressed during normogenesis. Further, the relative expression patterns of each subtype during each developmental time point were assessed. In order to have a deeper insight in the mechanism of endocrine disruption (for example, the up-regulation of vtg in embryos exposed to xenoestrogens), it is important to know, how the synthetic ligands, like EE2, regulate the expression of each ER subtype, therefore further research was dedicated to this issue.

In the study presented in chapter 3, the aim was to investigate where the three subtypes and especially the most abundant subtype (ERβ2) transcript are localized. Consequent to the discovery of ERβ2 in neuromasts, the question raised whether ERβ2 plays a role in the development of the lateral line organs. After having identified the role of ERβ2 in neuromast hair cell differentiation, the molecular mechanisms involved in the action of ERβ2 in zebrafish embryos were further investigated, revealing a crosstalk with the Notch signalling pathway and a role in neurogenesis.

In chapter 4, a review is presented where the knowledge gained from the chapter 3 was integrated in a bigger picture comprising a summary of studies that revealed functional genes needed for neuromast development linked with studies that assessed neuromast disruption as a result of toxicant exposure.

The appendix A contains two preliminary experiments, whose aim was to detect the dominant ER in the developing brain with two different in situ hybridization techniques. In the appendix B, the exposure of embryos to an ER antagonist is presented. The
1.2 Scope of the thesis

purpose was to phenocopy the ER$\beta_2$ morphants chemically in order to screen for potential new ER$\beta_2$ antagonists.

As ecotoxicology is challenged with different aspects of the developmental processes, this thesis contributed to open a new window to assess endocrine disruption on a molecular level as well as on a new biological endpoint.
CHAPTER 2

EARLY AND DOMINANT EXPRESSION OF ERβ₂ AND UP-REGULATION OF ERα UPON EXPOSURE TO EE₂ IN ZEBRAFISH (DANIO RERIO) EMBRYOS/LARVAE

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Abstract

Estrogens act on target genes mainly via the genomic pathway through binding to their nuclear receptors. In zebrafish, three subtypes of estrogen receptor (er) have been discovered and sequenced, erα, erβ₁ and erβ₂. Most of the studies to date have focused on their expression in adults. Here we show the pattern of expression of three subtypes using quantitative RT real-time PCR from 4 hpf to 120 hpf. Erβ₂ appeared to be mostly expressed and might therefore be responsible for mediating estrogen action in embryos/larvae. Between 4 and 8 hpf the high erβ₂ transcript levels measured could be related to maternally loaded mRNA. The decline measured at 12 hpf could be its degradation. Afterwards, the expression level constantly increased, which could be the start of the machinery of transcription. Besides natural ligands, nuclear receptors accept synthetic ligands that mimic estrogen actions. Here we show that EE₂, one of the most potent endocrine dis-
rupters, regulates each subtype differently. Surprisingly, **ERα**, the less expressed subtype, was clearly up-regulated. The two others were slightly or not affected. These results give an indication of how estrogen actions are mediated in early development and, moreover, help to understand endocrine disruption mechanisms in the early life stages of zebrafish.

### 2.1 Introduction

Estrogen is involved in physiological actions such as growth and differentiation, and supports the functioning of the reproduction system in males and females, as well as the normal functioning of the central nervous system (Duffy, 2006; Gruber et al., 2002; Hall et al., 2001). The molecular action of estrogens is mediated through two distinct types of signalling, often referred to as the genomic and the non-genomic pathways. The non-genomic pathway is characterized by its rapid physiological effects (Falkenstein et al., 2000; Watson and Gametchu, 2003; Revelli et al., 1998; Sutter-Dub, 2002; Hennessy et al., 2005; O’Lone et al., 2004). For example, estrogens are capable of modulating the physiology of nerve cells within seconds (Hall et al., 2001; Falkenstein et al., 2000). For the long-lasting effects such as the regulation of development and reproduction, estrogens take the well-studied genomic pathway, characterized by their binding to specific nuclear receptors, the so-called estrogen receptors (ERs). When estrogen diffuses into the cells, it binds to the ligand-binding domain of the receptor, allowing a conformational change. This complex enters the nucleus, where it binds to a specific sequence of DNA called estrogen responsive element (ERE) (Duffy, 2006; Gruber et al., 2002). In addition, ER can indirectly associate with promoters through protein-protein interactions with other DNA-binding transcription factors such as SP-1 (Stimulatory Protein) or AP-1 (Activator Protein), the so-called ligand independent action of ERs (O’Lone et al., 2004; Klinge et al., 2004; Kimbrel and McDonnell, 2003; Klinge, 2000). In both cases, ERs interact with various cofactors and enhance transcription of estrogen target genes.

The ERs are members of the nuclear steroid/thyroid hormone-receptor superfamily and share a common structure-function organization in different functional modules (Duffy, 2006; Nilsson et al., 2001; Gruber et al., 2002; Mennet et al., 2002). In mammals there are two subtypes, **ERα** and **ERβ**. Even if both modulate gene expression in a similar manner, some differences exist in their mode of action (Kuiper et al., 1997; Pettersson and Gustafsson, 2001; Duffy, 2006; Kimbrel and McDonnell, 2003). Moreover, they do not have the same activator intensity, nor do they respond to agonists in the same manner, and **ERβ** can modulate or attenuate the effects of **ERα** (Lindberg et al., 2003; Strom et al., 2004; Gougelet et al., 2007). For these reasons, it has been hypothe-
Early and dominant expression of ERβ2 and up-regulation of ERα

sized many times that ER subtypes have different biological functions (Pfaffl et al., 2001; Le Saux et al., 2006; Saunders et al., 2001; Hall and Korach, 2002). Moreover, a different role is attributed to each subtype, while ERα stimulates growth, ERβ suppresses proliferation (Pettersson and Gustafsson, 2001; Duffy, 2006; Klinge et al., 2004). Their different ligand binding affinities have also been shown with both natural and synthetic estrogens, and a unique tissue distribution pattern has also been described (Kuiper et al., 1997; Kimbrel and McDonnell, 2003; Routledge et al., 2000; Barkhem et al., 1998; Kuiper et al., 1998a).

One kind of synthetic ligand is the well studied 17α-ethinylestradiol (EE2), one of the most potent endocrine disruptors (Andersen et al., 2000, 2003; Nash et al., 2004; Van den Belt et al., 2002). It is known to bind to the ER and to variously affect reproduction in zebrafish, depending on exposure time and concentration Brion et al. (2004); Andersen et al. (2000); Colborn et al. (1993); Andersen et al. (2003); Van den Belt et al. (2002). It has been shown that early life stages are sensitive to low concentrations (Brion et al., 2004; Andersen et al., 2003) and that exposure during early development can permanently modify the organization of the reproductive, immune and nervous systems (Guillette et al., 1995; Bigsby et al., 1999; Andersen et al., 2000; Colborn et al., 1993; Papoulias et al., 2000; Andersen et al., 2003). The concentration and time of exposure used in these studies ranged from 1 to 25 ng/L and from 24 to 30 days (Van den Belt et al., 2002; Nash et al., 2004; Andersen et al., 2003; Bogers et al., 2006). Exposure studies with E2, the endogenous estrogen and EE2 revealed that they can reverse the sex ratio in favour of females and induce vitellogenin (precursor for the egg yolk proteins) (Brion et al., 2004; Andersen et al., 2000, 2003; Van den Belt et al., 2002). Contradictory results have been found depending on the organism and on the cell context studied. In the rat and mouse brain the erα and erβ transcript as well as the proteins were down-regulated by E2 (Patisaul et al., 1999; Agarwal et al., 2000). In the rat pituitary and cell lines, ER proteins were also suppressed by estrogens (Schreihofer et al., 2000). In the rat uterus, an up-regulation has been observed for the erα transcript and a down-regulation of the protein, depending on the cellular localization (Wang et al., 1999). In adult fish, many studies (mostly in livers) revealed a specific regulation for each subtype; in all studies erα mRNA was up-regulated (Filby and Tyler, 2005; Flouriot et al., 1997; MacKay et al., 1996; Sabo-Attwood et al., 2004; Islinger et al., 2003).

In teleosts, a second ERβ has been discovered and described in many species, including zebrafish (Ma et al., 2000; Hawkins et al., 2000; Menuet et al., 2002; Filby and Tyler, 2005; Gruber et al., 2002; Nelson et al., 2007). The zebrafish erα, erβ1 and erβ2 genes have been sequenced and are known to be similar to their human homologs (Menuet et al., 2002,
2.1 Introduction

A study using whole mount in situ hybridization revealed an early expression of \(er\beta\)s before 3 hpf, which corresponds to the maternally loaded mRNA. At the segmentation period (10-12 hpf) all subtypes were detected (Tingaud-Sequeira et al., 2004). In addition, weak \(er\alpha\) expression has been detected in an RNA protection assay experiment at 12 and 48 hpf, and it enhances dramatically at around 72 hpf, as well as for the two other subtypes (Bardet et al., 2002). For \(er\beta_2\) more information is available. A study using competitive reverse transcription (RT) PCR detected the highest level of expression in the zygote at 0 hpf (Lassiter et al., 2002). Two other studies confirmed the presence of maternally loaded mRNA, one before 3 hpf using RT real-time PCR and the other between 1 and 6 hpf using RNA protection assays (Tingaud-Sequeira et al., 2004; Bardet et al., 2002). All studies detected a decrease of expression until 12 hpf or even until 24 hpf by competitive RT PCR study. The embryonic start of transcription for \(er\beta_2\) is thought to be between 24 hpf and 48 hpf. A quantitative pattern of expression for all three genes has not been clearly described over all time points in zebrafish.

As already mentioned, the three subtypes are differently regulated by xenoestrogens. In zebrafish liver, \(er\alpha\) was induced, \(er\beta_1\) markedly reduced and \(er\beta_2\) was not regulated (Menuet et al., 2004). The \(er\alpha\) promoter has been characterized, and the presence of three half ERE sites and an imperfect ERE, as well as AP-1 and AP-4 sites (which could also be involved in the zebrafish \(er\alpha\) induction (Menuet et al., 2004)), was shown. The sequence of the \(er\beta_2\) promoter revealed two ERE half-sites, as well as SP1 binding sites, but after exposure to estrogen no response has been observed in the cell line used (ZF-4), perhaps because of the lack of appropriate cofactor expression for the SP1/ER interactions (Lassiter et al., 2002).

Very little is known about \(er\) expression and how they respond to endocrine disruptors during embryonic development in zebrafish. Therefore, it is urgent to clarify the pattern of expression of \(ers\) during normogenesis in zebrafish in order to identify a critical window where embryos/larvae could be most sensitive to endocrine disruption. It is, furthermore, necessary to clarify which of these receptor subtypes was most important during the early life stages. To understand mechanisms of endocrine disruption it is also essential to know how each subtype is regulated in response to a synthetic ligand. More specifically, both the up-regulation of \(er\alpha\) has to be confirmed and knowledge on how the \(er\beta\)s are regulated has to be obtained.
2 Early and dominant expression of ERβ2 and up-regulation of ERα

2.2 Materials and methods

2.2.1 Zebrafish maintenance

The fish facility was a recirculating flow-through system with tap water, which was sterilized with active carbon and UV light, set at 28°C. The zebrafish strain was a wildtype mix, mainly from petshops. The adults were kept in 75L glass aquaria and group matings were performed in a density of around 30 fish. Water is changed weekly and the room is set for a 14-hour-light-10-hour-dark cycles according to Westerfield (1995). Egg collectors with a black ground furnished with glass stones, sunk the night before the experiment, were used. The adult diet contained mostly live food, *Artemia nauplia* but also vitamins and dry food flakes. The eggs were collected one hour after the daylight. After pooling from the different aquaria, they were washed with a E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) and either exposed to chemicals in glass Petri dishes or raised in polystyrene Petri dishes until sample collection.

2.2.2 Sample collection

For the analysis of gene expression during normogenesis the eggs were separated in 9 groups of 60, one group for each time point (4, 6, 8, 12, 24, 48, 72, 96, 120 hpf) in three independent experiments (biological triplicates). When the embryos/larvae reached the age wanted, they were euthanized in MS222 (1g/L 3-aminobenzoic acid ethyl ester (Buchs, Switzerland), 600 mg/L NaHCO3, pH 7), incubated at 4°C overnight in RNAlater (Quiagen, Hombrechtikon, Switzerland) and stored at -20°C until RNA isolation.

2.2.3 EE2 exposure

The stock solution was made with 0.9 mg of 17α-ethinylestradiol (EE2, Sigma-Aldrich, Buchs, Switzerland) dissolved in 1ml 100% EtOH (puriss. p.a.; Fluka, Buchs, Switzerland) as solvent. The solvent control solution was made with 100 ml E3 and EtOH to a final concentration in the solution of 0.01%. To obtain the final dilution of 10, 50, 100, 1000, 2000 ng/L EE2, five previous dilutions were made at different concentrations in order to obtain a concentration of solvent which does not exceed 0.01% EtOH in a volume of 100 ml E3. All glassware was rinsed with Acetone and incubated overnight in exposure solution in order to saturate the glass surface with EE2. After fertilization (1 hpf) the eggs were distributed in glass Petri dishes at a density of 60 per 40 ml of solution. After 24 hpf the unfertilized eggs were removed and every 24 hpf the medium was renewed in order to maintain the EE2 concentration. The samples (around 30 embryos/larvae) were
2.2 Materials and methods

collected every 24 h, from 24 hpf to 120 hpf, and euthanized as well as stored as described above. Three independent experiments were performed.

2.2.4 Analytics

The stock solution concentration was measured by LC/MS/MS (TSI) as described in Vermeirssen (Vermeirssen et al., 2005). The stock solution was diluted to 40 ng/200µL. The analytical results corresponded well to the nominal concentrations (stock solution: 44.6 ng/200µl; 10 ng/L EE2: 11.6 ng/L; 50 ng/L EE2: 56.3 ng/L; 100 ng/L EE2: 107 ng/L; 1000 ng/L EE2: 1120 ng/L). The solvent control measurements were below detection limit.

2.2.5 RNA extraction

After the exposure experiment was performed and samples were collected, the total RNA was extracted from the embryos/larvae at each time point. Diethylpyrocarbonat (DEPC; Sigma-Aldrich, Buchs, Switzerland)-treated H2O was added to RNAlater in order to reduce the density of the solution in order to easily centrifuge the embryos. After centrifuging, the liquid was removed and replaced by the RLT-buffer (RNeasy Mini Kit (Quiagen AG, Hombrechtikon, Switzerland)) enriched with β-Mercaptoethanol (1:100). Homogenization was performed with a Polytron® (Kinematica AG, Switzerland). RNA was isolated with an RNeasy Mini Kit (Quiagen AG, Hombrechtikon, Switzerland). After elution, the total RNA was dissolved in 30 µl RNase-free H2O and the concentrations were measured with the Nanodrop spectrophotometer (Witeg, Littau, Switzerland). To control the quality and quantity of the extract, a 1% agarose gel was loaded with 500 ng RNA of each sample.

2.2.6 RT real-time PCR

A reverse transcription real-time polymerase chain reaction (RT real-time PCR) was performed to analyse gene expression in untreated and treated embryos/larvae. The total RNA (1200 ng) was first treated with DNase (Sigma-Aldrich, Buchs, Switzerland) to remove the co-extracted DNA. Pure RNA was reverse transcribed using ImPromII (Promega, Madison, WI, USA) in a final reaction volume of 20 µl primed with clamped oligo(dT)s (5'- (T)16VN-3') in accordance with the supplier’s recommendations. An H2O control was also DNase digested and reverse transcribed. Real-time PCR was carried out using Taqman® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA) with 50ng cDNA per reaction in an ABI Prism 7500 (Applied
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Biosystems). Primers and taqman probes were added at different final concentrations depending on the gene (Table 1). All samples were measured in triplicate on 96-well plates in 25 µl final reaction volume. Three different controls were measured: the H2O DNase digested and reverse transcribed, the not reverse transcribed DNase digested RNA, and H2O not digested and not reverse transcribed. The thermal cycle was set as follows: 2 min at 50°C, 10 min at 95°C, 40x (15s at 95°C, 1min at 60°C). After the first run, a 1% agarose gel was run as control for the amplified product.

2.2.7 Primers and Probes design

Primers and probes design was performed with Primer Express® (Software v2.0, Applied Biosystems). To ensure the specific amplification of cDNA, primers were designed on different exons (Table 2.1). The exons were identified with the database on http://www.ensembl.org/Danio rerio/exonview using the accession number: AB 037185 for erα, AJ 414566 for erβ1, AJ 414567 for erβ2. The unlabelled primers were synthesized by Microsynth (Balgach, Switzerland) (for the sequence see Table 2.1). The taqman probes labelled with FAM (5': 6-carboxyfluoresceine green) and TAMRA (3': 5, 6-carboxy-tetramethylrhodamine) were synthesized by MWG-Biotech (Eberberg, Germany) (for the sequence see Table 2.1). The homology of the region chosen for the primer and probe design of the target genes was less than 50%. The specificity of each primer pair with its probe was tested with plasmids in cross reactions. Each gene was cloned into pcDNA3.1/v5-HID-TOPO (Invitrogen, Switzerland) by Menuet et al. 2004. Each primer pair was specific for its gene. The efficiency of each primer pair with its probe was also tested with a dilution series of plasmid concentrations. The calculated efficiencies were used for normalization (see section 2.2.8).
2.2 Materials and methods

Table 2.1: Sequences, locations and final concentrations of real-time PCR primers and taqman probes of \( e\alpha, er\beta_1, er\beta_2 \) and \( \beta\)-actin.

<table>
<thead>
<tr>
<th>Gene (GB No)</th>
<th>Probe</th>
<th>sequence 5' → 3'</th>
<th>exons</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>( e\alpha )</td>
<td>taqman</td>
<td>CTGGTGCAATTTCATTCTGTCCAGTCC</td>
<td>6-7</td>
<td>100nM</td>
</tr>
<tr>
<td>AB 037185</td>
<td>primer forward</td>
<td>TGTGTGTCTCAAGCCACATCATCTC</td>
<td>6</td>
<td>300nM</td>
</tr>
<tr>
<td></td>
<td>primer reverse</td>
<td>ACTGCACCATGAAGTTGTCCCAT</td>
<td>7</td>
<td>300nM</td>
</tr>
<tr>
<td>( er\beta_1 )</td>
<td>taqman</td>
<td>ACGGGCTGAGGAGATGGGAACGACG</td>
<td>8-9</td>
<td>200nM</td>
</tr>
<tr>
<td>AJ 414566</td>
<td>primer forward</td>
<td>GACCAGGAGACCCACACAGT</td>
<td>8</td>
<td>900nM</td>
</tr>
<tr>
<td></td>
<td>primer reverse</td>
<td>CATGTCCTCTCCAAGCACTAGA</td>
<td>9</td>
<td>900nM</td>
</tr>
<tr>
<td>( er\beta_2 )</td>
<td>taqman</td>
<td>CGGCCCTCACAGTCACACAAACATCAGTAC</td>
<td>9</td>
<td>50nM</td>
</tr>
<tr>
<td>AJ 414567</td>
<td>primer forward</td>
<td>CTCACACAGGCACTCCTAAAC</td>
<td>9-10</td>
<td>900nM</td>
</tr>
<tr>
<td></td>
<td>primer reverse</td>
<td>CATCCTCCCAGAACACTACAGATCA</td>
<td>10</td>
<td>900nM</td>
</tr>
<tr>
<td>( \beta)-actin</td>
<td>taqman</td>
<td>CTTCCAGCTTTCCTCTGGGTATGGAA</td>
<td>1-2</td>
<td>100nM</td>
</tr>
<tr>
<td>AF 057040.1</td>
<td>primer forward</td>
<td>AGGTCAACCAATTGGCAAT</td>
<td>1</td>
<td>300nM</td>
</tr>
<tr>
<td></td>
<td>primer reverse</td>
<td>GATGTCCAGTCGACTTCACTC</td>
<td>2</td>
<td>300nM</td>
</tr>
</tbody>
</table>

2.2.8 Data evaluation

The real-time PCR data was evaluated using 7500 System SDS Software (Applied Biosystems). The cycle threshold (\( C_t \)) value was fixed to 0.06, the detection limit to 36 cycles, and the base line between 3 and 15 cycles. This data was exported to calculate the normalized gene expression with Q-Gene Core Module from Muller et al. (2002). The reference gene, \( \beta\)-actin, was used as an internal control for normalization. The principle of this method is that the mean normalized gene expression (MNE) is directly proportional to the amount of the RNA of the target gene relative to the amount of the reference gene (see equation 2.1). This method does not assume that the PCR amplification efficiencies of the target gene and the reference gene are equal, and takes into account the efficiency of each gene separately. The efficiencies were measured as described in chapter 2.2.7 and calculated with equation 2.2.

\[
MNE = \frac{(E_{\text{ref}})^{C_t \text{ ref}}}{(E_{\text{target}})^{C_t \text{ target}}}
\] (2.1)
where $E$ is the measured efficiency, $C_t$ is the cycle threshold value (the cycle at which the fluorescence rises appreciably above the background fluorescence).

$$E = 10^{-\frac{1}{\text{slope}}}$$  \hspace{1cm} (2.2)

where $E$ is the efficiency and slope is the slope of the regression curve calculated from the dilution series. $E_{\beta\text{-actin}} = 1.8$; $E_{\text{era}} = 2.05$; $E_{\text{er},\beta_1} = 2.06$; $E_{\text{er},\beta_2} = 1.93$.

### 2.2.9 Statistics

The statistical significance of relative mRNA expression between age and concentrations was determined by one-way ANOVA with $p \leq 0.05$. MNE data were log-transformed to achieve normality and in the graphs the untransformed data were represented. A Post-Hoc Tukey test was performed for the data between 8 hpf and all other time points if the effect of age was tested. Dunett’s test was carried out for EE$_2$ exposures with the solvent control as the control category. Values were reported as arithmetic means ± the standard deviation (SDEV). The analyses were computed using Statistica 6.0.

### 2.3 Results

#### 2.3.1 Normogenesis of the reference gene

The expression level of $\beta$-actin should stay constant in order to attribute the variation to the target genes. It was therefore important to monitor the behaviour of the reference gene during normal development. As an inverted measure of RNA level, the $C_t$ values were measured by RT real-time PCR. The $C_t$ values of $\beta$-actin were not always constant (Figure 2.1). The cycle threshold value for $\beta$-actin decreased significantly between 8 and 12, 24, 48 hpf. This means that the level of mRNA increased. The statistical analysis revealed that the most constant time points for the $C_t$ value of $\beta$-actin were between 48 hpf and 120 hpf.
2.3 Results

Figure 2.1: Cycle threshold value of β-actin measured during normal development of embryos/larvae over time (hpf, hours post fertilization) with RT real-time PCR. All standard errors were expressed as standard deviations (SDEV). Values are reported as arithmetic means ± SDEV of biological triplicates. One-way ANOVA was followed by the Post-Hoc Tukey test when \( p \leq 0.05 \) was performed between 8 hpf and all other time points. Significant differences were shown at 12, 24, 48 hpf. Statistical significance is indicated by * for \( p<0.05 \).

2.3.2 Normogenesis of the target genes

The pattern of expression of \( er\alpha, er\beta_1, er\beta_2 \) relative to β-actin was assessed in order to compare their levels of mRNA between each other and to understand their importance in early development. \( er\alpha \) and \( er\beta_1 \) pattern showed a trend to increase from 8 hpf and 24 hpf onward, respectively (Figure 2.2). \( er\beta_2 \) transcript levels showed a biphasic pattern: first a decrease from 4 and 8 hpf to 12 hpf, followed by an increase between 12 hpf and 96 to 120 hpf. The mRNA abundance was highest for \( er\beta_2 \), followed by \( er\beta_1 \) and, finally, \( er\alpha \) was the lowest expressed subtype.

One-way analysis of variance (ANOVA) followed by the Post-Hoc Tukey test was performed between 8 hpf and all other time points. For \( er\alpha \) significant differences were detected between 8 and 72, 96, and 120 hpf. For \( er\beta_2 \) there was a significant drop between 8 and 12 hpf. \( Er\beta_1 \) levels differed significantly between 8 hpf and 120 hpf.
2 Early and dominant expression of ERβ$_2$ and up-regulation of ERα

Figure 2.2: Normalised expression of erα, erβ$_1$ and erβ$_2$ during normal development of embryos/larvae over time (hpf, hours post fertilization), measured with RT real-time PCR and normalised against β-actin using Q-gene. All standard errors were expressed as standard deviations (SDEV). Values are reported as arithmetic means ± SDEV of biological triplicates. One-way ANOVA was followed by the Post-Hoc Tukey test when \( p \leq 0.05 \) was performed between 8 hpf and all other time points. Significant differences were identified at 72, 96, 120 hpf for erα; at 120 hpf for erβ$_1$ and at 12 hpf for erβ$_2$. Statistical significance is indicated by * for \( p < 0.05 \).

2.3.3 Regulation of the reference gene

In the same ways as for the normogenesis, it was important to check/establish if EE$_2$ exposure had an effect on the expression of the reference gene. The mRNA abundance of β-actin was not affected by the treatment (Figure 2.3). The represented fold induction was the C$_t$ value at the different concentrations compared to the solvent control. After one-way ANOVA, the Dunnett’s test, done at each time point separately against the solvent control, revealed a significant induction only at 48 hpf for 1000 ng/L EE$_2$ (* for \( p \leq 0.05 \)). The Tukey’s test for each concentration separately against 24 hpf revealed significant differences as shown in Table 2.2. Two-way ANOVA (with two independent variables, factors: age and treatment) revealed significant differences between age (\( p \leq 0.001 \)), but not between treatments (\( p \geq 0.05 \)).
2.3 Results

Figure 2.3: Cycle threshold value for $\beta$-actin relative to solvent control (0.01% EtOH) set to 1. Embryos/larvae were treated from 24 to 120 hpf (hours post fertilization) with 10, 50, 100, 1000, 2000 ng/L EE$_2$. Ct values were measured with RT real-time PCR. All standard errors were expressed as standard deviations (SDEV). Values were reported as arithmetic means ± SDEV of biological triplicates. One-way ANOVA was followed by two Post-Hoc tests. The Dunnett’s test done at each time point separately against the solvent control, revealed a significant difference at 48 hpf for 1000 ng/L. The Tukey’s test for each concentration separately against 24 hpf revealed significant differences showed in Table 2.2. Two-way ANOVA with age and treatment as independent variables revealed significant differences between age, but not between treatments. Statistical significance is indicated by * for $p \leq 0.05$. 
2 Early and dominant expression of ERβ₂ and up-regulation of ERα

Table 2.2: Statistical results after one-way ANOVA followed by the Post-Hoc Tukey’s test between 24 hpf and all other time points. Each concentration was tested separately. Significance was accepted at p ≤ 0.05 (*); n.s. = p ≥ 0.05.

<table>
<thead>
<tr>
<th>Age</th>
<th>EtOH</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>1000</th>
<th>2000 [ng/L EE₂]</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hpf</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>72 hpf</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>96 hpf</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
</tr>
<tr>
<td>120 hpf</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
</tr>
</tbody>
</table>

2.3.4 Regulation of the ers genes

The effects of EE₂ exposure on ero, erβ₁ and erβ₂ were measured to analyse the mRNA regulation of each er subtype during early development, from 24 to 120 hpf. Embryos/larvae were exposed to 0.01% EtOH as solvent control and to 10, 50, 100, 1000, 2000 ng/L EE₂. The effect of 0.01% EtOH was tested and revealed no significant change in the transcript level of ers and β-actin (data not shown). The represented fold induction was the mean normalised gene expression at the different concentrations compared to the solvent control (Figure 2.4). The gene expression levels were measured with RT real-time PCR and normalised against β-actin. Ero was induced over the whole concentration range of EE₂ tested (Figure 2.4). However, at some concentrations not all developmental stages were significantly affected by erα mRNA induction. This was the case at 10 ng/L for 120 hpf and at 50 ng/L and 100 ng/L for 72 hpf and 96 hpf. The pattern is changing at 1000 ng/L, larvae at all ages showed a strong induction of ero transcripts. Ero was induced by a factor of 38 at 24 hpf, 16 at 48 hpf, 11 at 72 hpf, 35 at 96 hpf and 43 at 120 hpf. At 2000 ng/L the trend is not significant for 24 hpf and 48 hpf, but erα is significantly induced by a factor of 8, 31 and 138 at 72 hpf, 96 hpf and 120 hpf, respectively.
2.3 Results

Figure 2.4: Fold induction of \( \textit{er}\alpha \) relative to solvent control (0.01% EtOH) set to 1. Embryos/larvae were treated from 24 to 120 hpf (hours post fertilization) with 10, 50, 100, 1000, 2000 ng/L EE\( \textsubscript{2} \). The normalised gene expression was measured with RT real-time PCR and normalised against \( \beta\)-actin using Q-gene. All standard errors were expressed as standard deviations (SDEV). Values were reported as arithmetic means ± SDEV of biological triplicates. One-way ANOVA followed by the Post-Hoc Dunnett’s test when \( p \leq 0.05 \) was performed for each time point between the solvent control and the treatment. Statistical significance is indicated by * for \( p < 0.05 \).
Early and dominant expression of ER\(_{\beta}2\) and up-regulation of ER\(_{\alpha}\)

\(Er\beta_1\) did not show a clear pattern of regulation (Figure 2.5). However, at 2000 ng/L EE\(_2\) at 24 hpf there was a significant inhibition of \(er\beta_1\). \(Er\beta_2\) was significantly inhibited for 10 and 100 ng/L EE\(_2\) at 24 hpf and for 1000 ng/L EE\(_2\) at 48 hpf (2.5).

![Figure 2.5: Fold induction of \(er\beta_1\) and \(er\beta_2\) relative to solvent control (0.01% EtOH) set to 1. Embryos/larvae were treated from 24 to 120 hpf (hours post fertilization) with 10, 50, 100, 1000, 2000 ng/L EE\(_2\). The normalised gene expression was measured with RT real-time PCR and normalised against \(\beta\)-actin using Q-gene. All standard errors were expressed as standard deviations (SDEV). Values were reported as arithmetic means ± SDEV of biological triplicates. One-way ANOVA followed by the Post-Hoc Dunnett test when p≤0.05 was performed for each time point between the solvent control and the treatment. Statistical significance is indicated by * for p<0.05.](image)
2.4 Discussion

Even if three *ers* are present in zebrafish, it is not clear if all three are equally mediating estrogen action. This work showed for the first time that each of these subtypes are variously expressed through embryo development. Although the three subtypes (*er*α, *er*β₁, and *er*β₂) were present since the beginning of the embryo development, *er*β₂ was the most expressed during the early life stages. Our results also illustrated that expression of the *er*α receptor was clearly enhanced (up-regulated) upon exposure to xenoestrogen (EE₂); expression of *er*β₁ and *er*β₂, on the contrary, was reduced (down-regulated) or not affected. Therefore, we show here that there is evidence for a different response of each *er* subtype to a synthetic ligand in zebrafish embryos/larvae.

In this study we were able to show for the first time the *ers* transcript levels during normogenesis in the early life stages of zebrafish. Ers transcripts have been assessed with different methods before, with a focus on *er*β₂ (Menuet et al., 2002, 2004; Bardet et al., 2002; Lassiter et al., 2002; Ma et al., 2000; Tingaud-Sequeira et al., 2004). Here we quantified all three *er* subtype transcripts with real-time PCR during the early development of zebrafish, which turned out to be a more sensitive method that the one used previously. We showed that all three *er* subtypes were already present at 4 hpf. The high level of *er*β₂ expression between 4 and 8 hpf could be interpreted as maternally loaded mRNA degraded at 12 hpf, as was described earlier (Tingaud-Sequeira et al., 2004; Bardet et al., 2002, Lassiter et al., 2002). The start of the machinery of transcription could be set in this case from 24 hpf onwards, supported by others (Bardet et al., 2002). The level of expression constantly increased afterwards. Since *β*-actin was used for normalization, its level of mRNA between 8 and 12 hpf has been checked. The results revealed an increase of *β*-actin transcript during this time, which indicates an underestimation of the possible *er*β₂ maternally loaded transcripts degradation. For the two other *er* subtypes, the expression pattern indicated that their expression level increased over time, which could be due to an increasing role over development. The increase of *β*-actin mRNA level between 12 hpf and 48 hpf is not yet understood, as the half life of *β*-actin is not known so far. One explanation could be that this increase is the result of the addition of endogenous transcripts to the maternal mRNA during this time. Its degradation would be then compensated by the increase of endogenous mRNA.

This study tells us that the three different subtypes have a different pattern in early life stage and that *er*β₂, being mostly expressed, might be the main player in mediating estrogen action in zebrafish embryos/larvae.

It is now well established that environmental estrogens mimic natural hormones by
binding to their natural receptors. What has still not been clarified is how these receptors are regulated by their natural or synthetic ligand in the early development in zebrafish. Here we show that in zebrafish embryos/larvae erα was up-regulated by EE2. In adult fish, it has already been mentioned that the natural ligand induced erα expression, suggesting an auto-regulatory mechanisms of this gene (Bowman et al., 2002; Pakdel et al., 1991; Flouriot et al., 1997; Menuet et al., 2004; Sabo-Attwood et al., 2004). This contrasts with other nuclear receptors that were inhibited by their ligand, as shown with glucocorticoids and progesterone receptors (Okret et al., 1986; Wei et al., 1988). Surprisingly, this negative feedback loop is not present in the erα-ligand pathway. For erβ1 and erβ2 we found similar results, as reported in adult zebrafish and fathead minnow liver studies (Menuet et al., 2004; Filby and Tyler, 2005). In both cases there was either a slight or no down-regulation in response to EE2. The choice of the β-actin gene for normalization appeared to be accurate. First, the transcript level stayed constant during the period of exposure (24 hpf to 120 hpf). And secondly, EE2 did not affect the transcription level of β-actin.

One of the explanations why the regulation of each er subtype varies to such an extent has to be related to the differences in the promoter sequence of each subtype. It is known that erα in zebrafish contains EREs in its promoter region. There are three half ERE sites and an imperfect ERE, as well as AP-1 and AP-4 (Menuet et al., 2004). Other transcription factors (Jun and Fos) can bind to these sites and also contribute to erα induction (Webb et al., 1999; Paech et al., 1997). No information is yet available regarding the promoter sequence of erβ1. The sequence of the erβ2 promoter revealed two ERE half-sites and SP1 binding sites. After exposure to E2, however, there was no regulation observed, perhaps because of the lack of appropriate cofactor expression for the SP1/ER interactions in the system used (Lassiter et al., 2002). From these results we can hypothesize that the presence of different EREs in the promoter region of the three subtypes and their different affinity to regulatory elements in the promoter region were able to dictate their specific responsiveness to E2 or EE2, which has also been concluded elsewhere (Gustafsson, 1999; Kuiper et al., 1998a; Flouriot et al., 1997).

There is evidence that ers regulation in response to exposure to E2 or EE2 is specific for each subtype. Furthermore, it has also been claimed that they do not contribute equally to the transcriptional regulation of genes (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006). For example it has been well established that E2 induce vitellogenin synthesis in fish (Bowman et al., 2002; Flouriot et al., 1997; Pakdel et al., 1991; MacKay et al., 1996). However, it is not clear if each ER subtype contributes equally to the transcriptional regulation of this gene. In this study the up-regulation of erα in embryos/larvae possibly correlates with the regulation of vitellogenesis during embryonic development described
2.4 Discussion

elsewhere (Muncke and Eggen, 2006). In the literature, it has been demonstrated many times that the mRNA induction of the vitellogenin gene correlated with the EE2 or E2-dependent up-regulation of erα gene expression (Menuet et al., 2004; Filby and Tyler, 2005; Sabo-Attwood et al., 2004). However, a very recent study in rainbow trout has shown contradictory results, where erβ was shown to be responsible for the vitellogenin induction (Leanos-Castaneda and Van Der Kraak, 2007).

An additional explanation for the relative regulation of the er genes is that there are specific ligand-binding affinities for each of the ERs in fish and mammals (Kuiper et al., 1997, 1998a; Barkhem et al., 1998). For example 17α-estradiol had a five times higher affinity for ERα protein than for the other receptor subtypes. Moreover, natural and synthetic agonists or antagonists induced distinct conformational changes in the tertiary structure of the ERs, which induced differential cofactor recruitment (Routledge et al., 2000). This leads to differences in each subtype’s transcriptional regulation, but also in the regulation of estrogen target genes.

These data show that the three er subtypes differ in transcript abundance in early development and inducibility by EE2, implying distinct physiological functions. Thus, there is a need to clarify the respective roles of each subtype in order to better explain the estrogen action in early development. This would in turn help to decipher the effects that xenoestrogens have on genes regulated via the ER pathways and, finally, to increase our understanding of the mechanism of endocrine disruption.

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CHAPTER 3

ESTROGEN RECEPTOR SUBTYPE $\beta_2$ IS INVOLVED IN NEUROMAST DEVELOPMENT IN ZEBRAFISH (DANIO RERIO) LARVAE

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Abstract

Estrogens are known to play a role in both reproductive and non-reproductive functions in mammals. Estrogens and their receptors are involved in the development of the central nervous system (brain development, neuronal survival and differentiation) as well as in the development of the peripheral nervous system (sensory-motor behaviours). In order to decipher possible functions of estrogens in early development of the zebrafish sensory system, we investigated the role of estrogen receptor $\beta_2$ (ER$\beta_2$) by using a morpholino (MO) approach blocking ER$\beta_2$ RNA translation. We further investigated the development of lateral line organs by cell-specific labelling, which revealed a disrupted development of neuromasts in morphants. The supporting cells developed and migrated normally. Sensory hair cells, however, were absent in morphants’ neuromasts. Microarray analysis and subsequent in situ hybridizations indicated an aberrant activation of the Notch signalling pathway in ER$\beta_2$ morphants. We conclude that signalling via ER$\beta_2$ is essential for hair
cell development and may involve an interaction with Notch signalling pathway during cell fate decision in the neuromast maturation process.

3.1 Introduction

A role of estrogens in the central nervous system (CNS) has been reported repeatedly (Duffy, 2006; Gruber et al., 2002; Hall et al., 2001). Besides sexual differentiation of the CNS, estrogens are involved in different aspects of brain development, as for example in neural differentiation and neuronal survival, or in the control of synaptic plasticity (Beyer, 1999; Meda et al., 2000; Nilsen et al., 2000; Wang et al., 2003). An increasing number of studies in mammals described estrogen functions that are not related to sexual activities, such as improvement of memory, blocking of apoptosis, maturation of motor behaviours and influence on the electrophysiological functions of neurons (Beatty and Holzer, 1978; McEwen and Alves, 1999). In zebrafish, a recent study showed that estrogens are essential for the proper developmental of sensory-motor behaviours, suggesting a novel role for these hormones in the peripheral nervous system (PNS) of fish (Nelson et al., 2008). The molecular action of estrogens is mediated through two distinct types of signalling, often referred to as the non-genomic and the genomic pathway. In the latter pathway, estrogens form a complex with specific nuclear receptors, the so-called estrogen receptors (ERs), which then function as transcription factors by binding to specific DNA sequences in the promoters of estrogen target genes (Duffy, 2006; Gruber et al., 2002).

In mammals, there are two ER subtypes, ERα and ERβ. The role of each ER has been analysed using knock-out mice deficient in ERα (ERαkos) and in ERβ (ERβkos) (Korach, 1994; Krege et al., 1998). Both knock-out animals exhibit distinct phenotypes, suggesting a distinct function for each ER (Gustafsson, 1999). ERα is mainly responsible for estrogen action in the uterus. ERβ may not be directly responsible for the regulation of reproductive physiology in rodents, but is suggested to mediate some of the many other effects of estrogens, such as neuronal survival and late embryonic development of the brain, as well as to have a function in memory and learning processes (Bodo and Rissman, 2006; Gustafsson, 1999; Kuiper et al., 1998b; Lindner et al., 1998; Osterlund et al., 1998). Interestingly, ERβko mice suffered from a severe hearing defect caused by disruption of hair cells of the inner ear, where ERs are also expressed in mouse and rat (Hultcrantz et al., 2006; Koenig, 2007; Stenberg et al., 1999).

Altogether, the non-reproductive roles of estrogens in the CNS and PNS, as cited previously, suggest that estrogen-dependent processes are broader than thought before. Therefore, research allowing increase in our understanding of estrogens physiological func-
Estrogen receptor subtype $\beta_2$ is involved in neuromast development

tions beyond the sexual system, is needed. In order to fulfill this gap, we investigated
the role of the ER in embryonic development of the model organism zebrafish. Like in
other teleosts, a second ER$\beta$ paralog has been identified in zebrafish (Filby and
Tyler, 2005; Gruber et al., 2002; Hawkins et al., 2000; Ma et al., 2000; Menuet et al.,
2002; Nelson et al., 2007). These subtypes were previously named $er\alpha$, $er\beta_1$ and
$er\beta_2$ and then renamed by the Zebrafish Information Network (ZFIN) (http://zfin.org)
to $esr1$, $esr2b$ and $esr2a$, respectively. In order to facilitate comparison with other
animals and previously published papers, we adopted the old nomenclature. In adult
brain, $er\beta_2$ transcripts have been detected in the two neuroendocrine regions, the
preoptic area and the mediobasal hypothalamus, and also in liver and gonads (Menuet et al.,
2002). In early embryos, $er\beta_2$ was the most expressed subtype and its highest level
was detected in freshly fertilized eggs, suggesting the presence of maternally loaded
mRNA (Lassiter et al., 2002; Tingaud-Sequeira et al., 2004). The embryonic start of
transcription for $er\beta_2$ is thought to be between 24 and 48 hours post fertilization
(hpf) (Bardet et al., 2002). $Er\beta_2$ mRNA is expressed during early life stage in the
epidermis, pectoral fin buds, hatching gland and developing brain. Surprisingly, $er\beta_2$
was also found to be expressed in the neuromasts, which are part of the lateral line
organs, the superficial organs of the sensory system (Tingaud-Sequeira et al.,
2004).

A rosette-like structure of neuromast is composed of two groups of cells, the
supporting cells and the hair cells, the latter closely related to the inner ear sensory
hair cells of mammals (Nicolson, 2005). This cluster of cells is innervated by sensory
neurons projecting to the CNS (Lopez-Schier et al., 2004; Metcalfe et al., 1985). The
function of lateral line organs has been reported to be mainly detection of directional
water movements, facilitating prey capture and predator avoidance. The lateral line is
divided into two major components: the anterior lateral line (ALL) which includes the
neuromasts of head, jaw and opercle, and the posterior lateral line (PLL) which includes
the neuromasts of the trunk and tail (Gompel et al., 2001).

Estrogen production enzyme, the aromatase, has been reported to be expressed in
neuromasts and to play a role in hair cell development (Kalivretaki et al. 2008, in prep).
Although ER$\beta_2$ mRNA was also detected in the supporting and hair cells of the mature
neuromasts (Tingaud-Sequeira et al., 2004), its role has not been investigated so far.
Therefore, the goal of this study was to examine whether ER$\beta_2$ has a role in neuromast
development. An elucidation of ER$\beta_2$ function in the lateral line organs would give an
indication of an estrogen action in the sensory system of zebrafish embryos and would
further add to the understanding of multiple functions that ERs are involved in during
various developmental processes.
ERβ2 knock down with a morpholino (MO) indicated a crucial function of ERβ2 in neuromast development. A more detailed analysis revealed that ERβ2 plays an essential role in hair cell differentiation. Moreover we suggest for the first time that ERβ2 might interact with the Notch signalling pathway during hair cell differentiation. Based on these findings, we hypothesized that ERβ2 is involved in neuromast development by interacting with the Notch signaling pathway.

3.2 Methods

3.2.1 Zebrafish maintenance

The zebrafish strain was a wildtype mix obtained from a petshop. The zebrafish were reared in a recirculating flow-through system with tap water treated with active carbon and UV light, set at 29°C. The adults were kept in 75L glass aquaria in a density of around 30 fishes. Water was renewed weekly and the room was maintained on a 14-hour-light-10-hour-dark cycle according to Westerfield (Westerfield, 1995). The adult diet contained mostly live food, Artemia nauplia but also vitamins and dry food flakes. The transgenic strain SqET4 was injected in Bettina Schmid’s laboratory (Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Alzheimer’s and Parkinson’s Disease Research, Ludwig-Maximilians-University, Munich, Germany).

3.2.2 Synthesis of DIG-labeled riboprobes

Erβ2 cDNA, comprising the complete coding region, cloned into pcDNA3.1/V5-His-TOPO, was kindly provided by Menuet et al. 2004. For the synthesis of a ribobpore corresponding to the A/B domain of the zebrafish erβ2, the relevant 429 bp fragment was amplified with PCR using primers fw: 5’-GTGTGGTTACCCGCCTTGCT-3’ and rev: 5’-TGAGGAACATGGCTGTGAG -3’, and cloned into pGemT-easy (Promega, Switzerland). For the sense ribobpore production, the plasmid was linearized with NcoI (Fermentas, Switzerland) and RNA was synthesized in vitro with Sp6 RNA polymerase (Promega, Switzerland). For the antisense riboprobe synthesis, the plasmid was linearized with SalI (Fermentas, Switzerland) and RNA was synthesized with T7 RNA polymerase (Promega, Switzerland). The plasmids containing claudinb (cldb) and keratin15 (k15) genes were linearized with NotI for cldb and with BamHI for k15. The antisense probe was synthesized using Sp6 RNA polymerase (Promega, Switzerland) for cldb and T7 RNA polymerase (Promega, Switzerland) for k15. The plasmid for ngn1 was kindly provided by B. Schmid, (Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for
Alzheimer’s and Parkinson’s disease Research, Ludwig-Maximilians-University, Munich, Germany). The plasmid was linearized with XhoI and T7 RNA polymerase was used for synthesis of the antisense probe. The plasmids for notch3 (=notch5) and notch1a were received from the group of Jiang in Singapore (Ma and Jiang, 2007). The plasmid was linearized with BsmHI and T3 RNA polymerase was used to synthesize the antisense probes. The DIG dNTPs (Roche, Switzerland) were used for probe labelling. Template DNA was removed by RNase free DNase (Promega, Switzerland) treatment.

### 3.2.3 Whole mount In Situ Hybridization (ISH)

The embryos were depigmented in 0.003% PTU (1-phenyl-2-thiourea, Sigma-Aldrich, Buchs, Switzerland) in E₃ medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33 mM MgSO₄) after 24 hpf. The larvae were euthanized in MS222 (1g/L 3-aminobenzoic acid ethylester (Sigma-Aldrich, Buchs, Switzerland), 600 mg/L NaHCO₃, pH 7) at 72 or 96 hpf and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS: NaCl 137mM, KCL 2.7mM, PO₄ 0.02M) at 4°C. After dehydration in 100% MeOH, larvae were stored at -80°C. Prior to use, they were rehydrated with 75%, 50% and 25% MeOH/PBS and rinsed three times 5 min with PBT (PBS plus 0.1% Tween20 (polyoxyethylene-sorbitan monolaurate; Sigma-Aldrich, Buchs, Switzerland)). To permeabilize the larvae, proteinase K (5µg/ml in PBT) digestion was performed for 30 min followed by a refixation in 4% PFA in PBS for 20 min. After washing several times in PBT, larvae were prehybridized for 4 h in HYBE (50% formamide (Fluka, Switzerland), 5x SSC, heparin 50mg/ml, 0.1% Tween20, tRNA 10mg/ml, citric acid 1M) at 70°C for erβ₂, 65°C for cldβ, ngn1, notch3, notch1a and 59°C for k15. For hybridization, the labeled riboprobes were added to the pre-warmed HYBE (1:400) and larvae were incubated overnight at 70°C. After washing at 70°C two times for 30 min in 50% formamide/50% 2xSSC plus 0.1% Tween20, the larvae were rinsed in 2xSSC plus 0.1% Tween20 for 15 min, and then two times in 0.2xSSC plus 0.1% Tween20 for 30 min. The larvae were then transferred into blocking buffer (1x PBS, 0.1% Tween20, 100mg/ml bovine serum albumin (BSA), 1% DMSO) to be blocked at room temperature for 3-4 h. The larvae were then incubated overnight at 4°C in a solution with preabsorbed sheep anti-digoxigenin-AP Fab fragments (Roche, Switzerland) at a 1:4000 dilution in blocking buffer. Before being transferred in staining buffer (0.1M Tris-HCl pH 9.5, 0.05M MgCl₂, 0.1M NaCl, 0.1% Tween20) in 6-well plates, the larvae were washed several times in PBT. Afterwards, the staining buffer was replaced with staining buffer plus 75mg/ml NBT (Nitro blue tetrazolium chloride, Roche, Switzerland) and 50mg/ml BCIP (5-Bromo-4-chloro-3-
indolyl phosphate, toluidine salt, Roche, Switzerland) and the larvae were stained until a signal was detected. Finally, the larvae were washed several times in PBT and mounted in 60% glycerol (Fluka, Switzerland) on glass slides to be observed under bright field microscope or with differential interference contrast filter (Leica, Switzerland).

3.2.4 Microinjection

Prior to injections, the black ground egg collectors were sunk into the different aquaria. The first pool of eggs was collected after 15 min, 30 min later the second pool of eggs was collected. The eggs were washed with E3 before and after injection. Injections were performed into the yolk of fertilized zebrafish eggs between the one- and four-cell stages. The injections were done using a PV820 Pneumatic Picopump (World Precision instruments, USA). The embryos were raised in E3 in petri dishes at 28℃.

3.2.5 Morpholinos and capped RNA

The ERβ2 morpholino (ERβ2MO) was designed against the flanking sequence to the ATG start codon of zebrafish crβ2 with the sequence 5’-ACATGGTGAAGGCGGATGAGTTCA G-3’. A standard control MO (coMO) 5’-CCTCTTACCTCAGTTACAATTTATA-3’ and a p53 MO 5’-GCGCCA TTGCTTTGCAAGAA TTG-3’ were used as controls for phenotype specificity. The MOs were obtained from Gene Tools, LLC (Philomath, oregon, USA) and diluted for injection in 0.01% DEPC (Fluka, Switzerland) water with Phenol Red (0.005%) as injection indicator. ERβ2 MO and coMO were injected at a concentration range between 15 µM and 100 µM. p53 MO was co-injected with ERβ2 at a concentration of 15 µM for both. ERβ2 capped RNA (cRNA) mutated in the MO binding region was co-injected with the ERβ2 MO in a concentration of 300 ng and 15 µM, respectively. For the synthesis of ERβ2 cRNA an insertion of 9 nucleotides into the complete crβ2 coding cDNA obtained from Menuet (Menuet et al. 2004) was performed with QuickChange® Site-Directed Mutagenesis kit (Stratagene, Switzerland). Primers were designed using Stratagene web page (http://www.stratagene.com/newline/qcprimerdesign). In a first step, 3 nucleotides (GAA) were introduced with primers fw: 5’-CTGAACTCATTCCGCCGAATT CACCATGTCCAG-3’ and rev: 5’-CTCGGACATGGGTAAATCGGCCGATGAGTTCC AG-3’. In a second step, additional 6 nucleotides (GAATTC) were inserted with primers fw: 5’-CTGAACTCATTCCGCCGAATT CACCATGTCCAG-3’ and rev: 5’-ACTCGGACATGGGTAAATCGGCCGATGAGTTCC AG-3’. For in vitro RNA synthesis, template DNA was linearized with NotI, the reaction was then purified with the MinElute Reaction Cleanup kit (Quiagen AG, Hombrechtikon, Switzerland). The cRNA
3 Estrogen receptor subtype $\beta_2$ is involved in neuromast development

was synthesized using mMessageMachine® kit (Ambion, Switzerland) and its concentration was measured with a Nanodrop spectrophotometer (Witeg, Littau, Switzerland).

3.2.6 Morphological and behavioural observations

Observation of injected embryos began at 24 hpf and was repeated every 24 h until 120 hpf. The embryos were observed under a stereomicroscope (Olympus Schweiz AG, Volketswil, Switzerland). Hatching rate and swimming behaviour were recorded between 48 hpf and 72 hpf. Behavioural tests were conducted to observe swimming behaviour and responses to a gentle touch of the tail with a needle at 72 hpf. The larvae that were not able to rapidly traverse the petri dish in a straight line and were swimming in circles were described as "larvae swimming in circles". Each larva was tested three times and, when it failed three times to swim straight, it was recorded as positive for swimming in circles. Finally, the number of larvae swimming in circles was expressed as percentage of the total number of alive larvae in the petri dish.

3.2.7 The staining of lateral line hair cells

The vital dye N-(3-triethylammoniumpropyl)-4-(dibutylamino)styryl) pyridinium dibromide (FM®1-43; Invitrogen, Switzerland) was used to label physiologically active hair cells within the neuromasts. The larvae were incubated for 30 sec in 3 $\mu$M of FM®1-43, washed several times with E3, and anaesthetized in MS222 (200 mg/L 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, Buchs, Switzerland), pH 7) at 72 hpf. They were then mounted in 3% methylcellulose (Fluka, Switzerland) and placed on their lateral side. The stained neuromasts were recorded. Imaging was processed with a green fluorescent protein (GFP) filter under a Fluorescent light microscope (Leica Microsystems, Heerbrugg, Switzerland).

3.2.8 Statistics

The statistical significance of the number of neuromasts of at least 35 individuals between uninjected embryos (blank) and ER$\beta_2$ MO-injected was determined by one-way ANOVA with $p=0.05$. A Post-Hoc Tukey test was performed to identify significant differences between the number of neuromasts in injected embryos and controls. All statistical analyses were computed using Statistica 6.0 (Statsoft, Inc., USA). Values were reported in box-plots performed with SigmaPlot (Systat Software Inc., California, USA).
3.3 Results

3.2.9 Microarray analysis

Transcriptional profiling was performed using affymetrix microarrays at the Functional Genomic Center in Zürich, Switzerland (http://www.fgcz.ethz.ch). The spotted array contains 14,900 oligonucleotides (Affymetrix Inc., USA). Sequence information for this array was selected from the following public data sources: RefSeq (July 2003), GenBank (release 136.0, June 2003), dbEST (July 2003), and UniGene (Build 54, June 2003). Zebrafish embryos were injected with MOs before RNA isolation as described above. Total RNA of 72 hpf ER$_{\beta 2}$MO- and coMO-injected embryos, as well as of uninjected embryos, was extracted using RNeasy mini kit (Qiagen, Switzerland). Concentration and purity of the isolated total RNA were assessed using Nanodrop spectrophotometer (Witeg, Littau, Switzerland) and gel electrophoresis. The RNA was prepared using the biotin-labelling IVT Kit (Affymetrix Inc., USA) prior to hybridization done with fragmentation buffer (Affymetrix Inc., P/N 900720), hybridization controls and control oligonucleotide B2 (Affymetrix Inc., P/N 900454) according to the Affymetrix gene expression protocol. After hybridization of the labelled RNA with the oligos on the microarray surface, the array was washed using Affymetrix Fluidics Station 450 (FS450 0004 protocol) and subsequently scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Inc.) according to the protocol of the manufacturer. Raw data processing was performed using the Affymetrix AGCC software. After hybridization and scanning, probe cell intensities were calculated by means of the MAS5 algorithm. The data were then analysed with Genespring (Agilent, USA). The data were normalized per chip (50th percentile) and per gene (to mean). Changes were identified by selecting transcripts with a fold change of $>2$ for increases and $<0.5$ for decreases. As a second criteria, only the genes found to be differentially expressed in both arrays "ER$_{\beta 2}$ MO at 15 µM" and "ER$_{\beta 2}$ MO at 50 µM" were selected as being affected by the ER$_{\beta 2}$ MO.

3.3 Results

3.3.1 er$_{\beta 2}$ mRNA presence in neuromasts

Er$_{\beta 2}$ transcripts were localized in 96 hpf embryos using whole mount ISH with a sense and an antisense probe against er$_{\beta 2}$ mRNA. The presence of er$_{\beta 2}$ mRNA was detected in the neuromasts of the ALL and PLL, when using antisense probe (Figure 3.1 A, B, C). In the head, ALL expression is difficult to identify because of increased background staining, also detected using the sense er$_{\beta 2}$ probe (Figure 3.1 D, E, F). Neuromasts of the PLL were clearly visible in higher magnification (Figure 3.1 C). Beside er$_{\beta 2}$, the two
Estrogen receptor subtype $\beta_2$ is involved in neuromast development. Other subtypes’ transcripts, $er\alpha$ and $er\beta_1$, were also localized with ISH, however, only weak expression in the head region and in the neuromasts has been detected (data not shown).

Figure 3.1: Localization of $er\beta_2$ mRNA with whole mount in situ hybridization in 96 hpf larvae. (A-C) Antisense probe was detected mainly in the brain and in the neuromasts (see black arrowheads). (D-F) With the sense probe, the signal was not detected in the neuromasts, but weakly in the brain. (A, B, D, E) Magnification 10x, scale bar = 200 $\mu$m. (C, F) Magnification 60x, scale bar = 50$\mu$m.
3.3 Results

3.3.2 ER\(\beta_2\) knock down phenotypes as a function of MO concentration

Expression of \(er\beta_2\) was blocked by injecting zebrafish eggs with a MO against ER\(\beta_2\) mRNA (ER\(\beta_2\) MO). The effects observed were MO-concentration-dependent and were described as curved tail, lower hatching rate, disrupted neuromast development and circular swimming behaviour. For schematic representation, these effects are plotted as a function of the MO concentration and the age of the morphants (Figure 3.2). No effects were observed when a standard coMO was injected at the same concentration range.

In embryos injected with 100 \(\mu\)M MO the hatching rate decreased to 15\% at 72 hpf compared to nearly 100\% in controls. In addition to reduced hatching rate, 10\% of the morphants receiving the high concentration of 100 \(\mu\)M MO developed a curved tail. The morphants did not survive longer than 96 hpf, independent of being hatched or not. Also at 50 \(\mu\)M MO the hatching rate was affected, as only 50\% of the embryos were able to hatch at 72 hpf compared to nearly 100\% in controls. Similarly to the 100 \(\mu\)M MO injected embryos, the embryos did not reach the age of 96 hpf. At 25 and 15 \(\mu\)M all embryos could hatch normally. At all MO concentrations tested, we could observe an alteration of the swimming behaviour and a reduction of neuromasts number, the latter being assessed with a specific hair cell staining, FM1-43.
3.3.3 The number of functional neuromasts is reduced in ERβ₂ morphants

In order to assess the occurrence of neuromasts, functional hair cells were stained with a vital dye (FM1-43) in 72 hpf embryos (Figure 3.3). After injection with 15 µM ERβ₂ MO, we observed a significant reduction in number of stained hair cells. In the coMO-injected embryos and in the uninjected ones (blanks), the mean number of stained neuromasts
3.3 Results

was 13.1 ± 2.13 (N=38) and 16.8 ± 2.76 (N=18) respectively (Figure 3.4 A). The mean number of neuromasts stained in the ERβ2 morphants was reduced to 3.7 ± 2.08 (N=46). When ERβ2 MO was co-injected with ERβ2 cRNA, the phenotype was rescued, although partially, to a mean number of neuromasts of 10.6 ± 5.05 (N=58) instead of 3.71 ± 2.08 (N=66) in the ERβ2 MO injected embryos (Figure 3.4 B). Injection of higher cRNA concentrations caused embryos to die, and hence the numbers of rescued embryos could not be increased. Contrary to the morphants, the rescued larvae developed functional neuromasts in the ALL and the PLL, reinforcing the specificity of knockdown phenotype (Figure 3.3 G-I).

The p53 gene is known to be activated upon injection of several MOs resulting in off-
Estrogen receptor subtype $\beta_2$ is involved in neuromast development targeting effects (Langheinrich et al., 2002), and its knock down is therefore an additional control for phenotype specificity. To exclude p53-provoked off-targeting effects as a reason for the ER$\beta_2$ MO morphant phenotype, we co-injected embryos with ER$\beta_2$ MO and p53 MO. The morphant phenotype did not change, the mean number of neuromasts in the co-injected embryos was at $3.32 \pm 2.5$ (N=44), compared to $3.8 \pm 2.9$ (N=44) in the treated embryos at 72 hpf (Figure 3.4 C). Thus, the results of co-injection of p53 MO and ER$\beta_2$ MO eliminated the eventual off-targeting effects and reinforced the specificity of the ER$\beta_2$ morphant phenotype.

Our results showed that ER$\beta_2$ is essential for the normal development of functional neuromasts. As a next step we investigated the presence and development of hair cells and supporting cells, which compose the neuromast, in the ER$\beta_2$ morphants.
3.3 Results

Figure 3.4: Number of neuromasts assessed using live staining (FM1-43) in wild type (A-C) at 72 hpf and with GFP visualisation in transgenic line (ET4) (D). The boxes symbolize the interquartile space of each distribution; the median is indicated with a horizontal line within the box, and dots indicate extreme values (5th/95th percentile). The number of individuals recorded for elaboration of the box plots is indicated with N=number of individuals. Numbers of neuromasts are shown in (A) ER\(_{\beta2}\) MO- or coMO-injected wild-type larvae (both MOs at 15 \(\mu\)M); (B) ER\(_{\beta2}\) MO-injected wild-type larvae (15 \(\mu\)M) co-injected with cRNA (300 ng); (C) ER\(_{\beta2}\) MO-injected wild-type larvae co-injected with p53 MO (both at 15 \(\mu\)M). (D) ET4 larvae injected with ER\(_{\beta2}\) MO or coMO (both at 15 \(\mu\)M). One-way ANOVA following the Tukey test were performed to test the differences between blank (uninjected) and injected embryos and significances were indicated with * when p<0.05.

3.3.4 Fewer hair cells are developed in ER\(_{\beta2}\) morphants

In order to investigate if ER\(_{\beta2}\) plays a role in the development of the hair cells of the neuromast, the ER\(_{\beta2}\) MO was injected into a transgenic line (ET4) expressing GFP in
3 Estrogen receptor subtype $\beta_2$ is involved in neuromast development

the hair cells (Parinov et al., 2004). GFP expression was reduced in the neuromasts of ER$\beta_2$ MO injected ET4 larvae (Figure 3.4 D). There are 2.2 ($\pm$ 1.8) neuromasts in ER$\beta_2$ morphants, in comparison with 13.7 ($\pm$1.4) in coMO and 13.9 ($\pm$ 1.7) in blanks. These results confirmed that the reduced FM1-43 staining observed in morphants corresponded to an absence of hair cells in neuromasts and showed that normal hair cell development is disturbed in ER$\beta_2$ MO treated embryos. The alteration of swimming behaviour gave further indications of the hair cell disruption in morphants. Normally, zebrafish embryos swim straight away when mechanically stimulated (needle). The embryos injected with all concentrations of ER$\beta_2$ MO showed an abnormal response when stimulated; the morphants were swimming in circles. The average of three biological replicates is plotted in Figure 3.5. Of the larvae missing ER$\beta_2$ (ER$\beta_2$ morphants), 93% ($\pm$ 4.7) were swimming in circles and showed an abnormal response to tail stimulus when injected with 15 $\mu$M of ER$\beta_2$ MO. This phenotype could be rescued after co-injection of ER$\beta_2$ MO with the ER$\beta_2$ cRNA and was not rescued after co-injection with the p53 MO.
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Figure 3.5: Percentage of larvae that were swimming in circles after injection with the ERβ2 MO or with a coMO at different concentrations (15, 25, 50, 100 µM) or uninjected (blank) at 72 hpf and 96 hpf. Values are reported as arithmetic means ± standard deviation of biological triplicates.

3.3.5 Presence of supporting cells in ERβ2 morphants

Besides the hair cells, the neuromasts are composed of two types of supporting cells, the sustentacular cells and the mantle cells. The ability of the morphants to develop supporting cells was investigated. To assess the presence of supporting cells in normal larvae and in ERβ2 morphants, we used cellular markers specific for supporting cells. ISH was performed against keratin15 (k15) to detect mantle cells (data not shown) and claudinb (cldb) to detect supporting cells (Lopez-Schier et al., 2004). The results showed an expression of cldb in the ALL as well as in the PLL of larvae injected with 15 µM ERβ2 MO (Figure 3.6). After ISH with the probe against k15, mantle cells were visible in morphants injected with 15 µM ERβ2 MO until the tip of the tail (Figure 3.6).

As a next step in unraveling the molecular mechanisms underlying the disturbed neu-
Estrogen receptor subtype $\beta_2$ is involved in neuromast development

In ER$\beta_2$ MO injected embryos, gene expression levels were examined using affymetrix gene arrays.

Figure 3.6: Detection of sustentacular cells and mantle cells with whole mount in situ hybridization against keratin15 ($k15$) (A, D) and claudinb ($cldb$) (B, C, E, F) in 72 hpf larvae injected with 15 $\mu$M ER$\beta_2$ MO (A-C), and with 15 $\mu$M coMO (D-F). (A, B, D, E) Magnification 10x, scale bar = 500 $\mu$M, Arrowheads point to neuromasts. (C, F) Magnification 100x, scale bar = 10 $\mu$M. Arrowheads point to visible hair cells.
3.3 Results

3.3.6 Up-regulation of pro-neural genes in ER$\beta_2$ morphants

We aimed at investigating the genes affected by ER$\beta_2$ suppression in early development by analysing modified gene expression levels in morphants using microarrays. Two independent affymetrix microarray sets were performed. One set comprised samples of 15 $\mu$M ER$\beta_2$ MO injected embryos, 15 $\mu$M coMO injected embryos and uninfected embryos. The second set comprised analogue samples at 50 $\mu$M. In the first set, using the total RNA from 50 $\mu$M ER$\beta_2$ MO injected larvae, 2666 genes were differentially expressed when comparing with larvae injected with 50 $\mu$M coMO. Twenty-one genes were differently expressed between blank (uninjected) RNA and RNA from 50 $\mu$M coMO injected larvae. These genes were mostly hypothetical proteins with unknown function. In the second array, using total RNA from 15 $\mu$M MO injected larvae, 1046 genes were differentially expressed when compared with larvae injected with 15 $\mu$M coMO. After comparing the list of genes differentially expressed in morphants injected with 15 $\mu$M and 50 $\mu$M ER$\beta_2$ MO, 631 genes were found to be affected by both MO concentrations in larvae.

In order to assign the affected genes into functional classes based on the gene ontology, information was taken from public databases using NCBI website (http://www.ncbi.nlm.nih.gov/sites/entrez). We classified the genes in 10 different functional groups, namely: egg and embryo development, energy metabolism, intra- and intercellular signalling, transcription and translation, cell growth and development, cell structure and cytoskeletal organization, detoxification and defence, intra- and intercellular transport and unknowns (Table 3.1). Of the differentially expressed genes, 45% had an unknown function, 18% were involved in energy metabolism, 10% were involved in transcription and translation and another 10% in intra- and intercellular transport, 2.6% of the genes were involved in embryo development. The raw data were submitted to GEO (Gene Expression Omnibus) website with the accession number GSE13771, (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE13771).
Table 3.1: Differentially expressed genes at 72 hpf in 15 μM ERβ2 MO and in 50 μM ERβ2 MO injected embryos, divided in functional classes.

<table>
<thead>
<tr>
<th>Gene description</th>
<th>number of genes</th>
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<tbody>
<tr>
<td></td>
<td>up-regulated</td>
<td>down-regulated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&gt; 2)</td>
<td>(&lt; 0.5)</td>
<td></td>
</tr>
<tr>
<td>egg and embryo development</td>
<td>17</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>energy metabolism</td>
<td>118</td>
<td>25</td>
<td>93</td>
</tr>
<tr>
<td>intra- and intercellular signalling</td>
<td>54</td>
<td>17</td>
<td>37</td>
</tr>
<tr>
<td>transcription and translation</td>
<td>67</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>cell growth and development</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>cell structure and cytoskeletal org</td>
<td>11</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>detoxification and defense</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>intra- and intercellular transport</td>
<td>66</td>
<td>14</td>
<td>52</td>
</tr>
<tr>
<td>unknown</td>
<td>285</td>
<td>128</td>
<td>157</td>
</tr>
<tr>
<td>TOTAL</td>
<td>631</td>
<td></td>
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</table>

Based on the combination of microarray and literature data, interesting genes involved in neurogenesis were selected. The *ngn1* gene was 2.6x and 7.8x up-regulated in 15 μM and 50 μM ER MO injected embryos, and the *nrd* gene was 2.2x and 3.3x up-regulated in 15 μM and 50 μM ERβ2 MO injected embryos, respectively. The *ngn1* gene was selected for more detailed analysis. ISH in ERβ2 morphants confirmed a differential expression of the *ngn1* gene in the morphants, where the *ngn1* expression pattern was disrupted (Figure 3.7). In morphants *ngn1* was expressed in the motor neurons, contrary to coMO-injected larvae, where *ngn1* expression was detected in neuromasts, corresponding to the sensory neurons. Expression of *ngn1* was higher in morphants’ heads than in heads of control larvae.
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Figure 3.7: Localization of *ngn1* mRNA with whole mount *in situ* hybridization in 72 hpf larvae. (A) In 50 µM ERβ2 MO-injected embryos; (B-C) in 15 µM ERβ2 MO injected embryos; (D) in 50 µM coMO injected embryos; (E, F) in 15 µM coMO injected embryos. (A, B, D, E) Magnification 10x, scale bar = 500 µm. (C) Magnification 60x, scale bar = 20 µm. (F) Magnification 100x, scale bar = 20 µm. Arrowheads point to *ngn1* expression.

Neuromast differentiation follows lateral inhibition mechanisms (Itoh et al., 2003). In this cell fate decision process in neuromasts, the *notch* gene has been described to play a role. We therefore searched for differential expression of Notch family members in morphants. Microarray data showed that *notch1a* was 3.6x and 4.3x up-regulated in 15 µM and 50 µM ERβ2 MO injected embryos respectively. The *notch3* gene was 2.2x up-regulated in 50 µM ERβ2MO injected embryos. Even if the induction of *notch3* has not been found in the 15 µM ERβ2 MO, ISH analysis showed differential expression of the *notch3* gene in morphants injected with 15µM ERβ2 MO (Figure 3.8 A-D). More *notch3* staining was detected in supporting cells of the morphants compared to controls. ISH with a probe against *notch1a* gene detected no clear up-regulation of mRNA staining in supporting cells of morphants injected with 15 µM ERβ2 MO (Figure 3.8 E-F).
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3.4 Discussion

ERs are known to play a major role in reproduction in vertebrates. They are also involved in diverse non-reproductive processes. In zebrafish larvae, ERs were found to be expressed in neuromasts (Tingaud-Sequeira et al. (2004); present study), however, their function in these lateral line organs remained unclear. Current work used a MO knock
3.4 Discussion

down technique to investigate the role of ERβ2 in the early development of zebrafish larvae, and particularly in neuromasts. Of the three ER subtypes present in zebrafish, ERβ2 exhibits the highest expression during early development (Bardet et al. (2002); Lassiter et al. (2002); Tingaud-Sequeira et al. (2004); Froehlicher et al., unpublished). The knock down of two other subtypes, ERα and ERβ1, was not performed in this work. We cannot exclude that ERβ2 action may be taken over by the two other receptors in ERβ2 morphants, however, severity, dose- and time-dependency, as well as specificity of the observed morphant phenotype suggest that this is not the case. Thus, although we cannot draw any conclusion regarding the possible involvement of ERα and ERβ1 in neuromast development, the results of our work provide a strong evidence for an important role of ERβ2 in this process.

ERβ2 is involved in hair cell development

The neuromasts are superficial sense organs of the mechanosensory lateral line, a sensory system closely related to that of the inner ear of mammals (Nicolson et al., 1998). Neuromasts develop from pro-neuromasts deposited by primordium migrating from head to tail. They comprise a cluster of cells which are innervated by sensory neurons projecting to the CNS (Lopez-Schier et al., 2004; Metcalfe et al., 1985). Hair cells form the core of the neuromast, surrounded towards periphery by the supporting cells of two types, sustentacular and mantle. Supporting cells constantly proliferate and are able to differentiate into hair cells, creating a continuous turnover of hair cells (Williams and Holder, 2000). This potential for the hair cells to regenerate may explain the phenomenon of hair cells recovery in the neuromasts of ERβ2 morphants after MO dilution with time.

A significant reduction in the number of neuromasts stained with vital dye FM1-43, marking functional hair cells in these organs, was observed in the ERβ2 morphants. This endpoint was dose-dependent (Figure 3.2) and ERβ2-specific, as it was not observed after injection of coMO and could be partially rescued by co-injection of ERβ2 cRNA (Figure 3.3 and 3.4). It was also not caused by off-targeting effects brought about by possible p53 induction, as co-injection of p53 and ERβ2 MOs did not protect the morphants from reduction of number of stained neuromasts. Our further experiments were designed to characterize the cell type affected in the neuromasts of ERβ2 morphants more specifically and to elucidate the possible reasons leading to this disruption.

Injection of ERβ2 MO into transgenic line ET4 expressing GFP in the hair cells of neuromasts led to significant reduction of GFP fluorescence (Figure 3.4), providing further evidence that hair cells were not only non-functional, but also not differentiated in
Estrogen receptor subtype \( \beta_2 \) is involved in neuromast development

neuromasts of morphants, as ET4 GFP labeling is a marker of differentiated hair cells in zebrafish neuromasts (Parinov et al., 2004; Lopez-Schier and Hudspeth, 2005; Sarrazin et al., 2006). At the same time, with use of supporting cell markers we could show that the primordium migration was not disrupted in ER\( \beta_2 \) morphants, and supporting cells of both types were present in neuromasts deposited until the tip of the tail (Figure 3.6). Taken together, our data show that the neuromast disruption in ER\( \beta_2 \) morphants concerned exclusively the hair cells of these organs, strongly arguing for the role of ER\( \beta_2 \) in hair cell development. Another recent investigation has shown the presence of estrogen production in neuromasts, using ISH to detect the expression of aromatase cyp19a1 in these organs and MO knock down to show its indispensability for the differentiation of hair cells (Kallivretaki et al., in preparation). Thus, locally produced estrogens may play a role in the development of the zebrafish sensory hair cells in the neuromasts, most probably through the classical genomic pathway mediated by ER\( \beta_2 \).

An interesting characteristic observed in ER\( \beta_2 \) morphants was an alteration of swimming behaviour: the larvae were unable to swim straight and performed circling movements instead. This biological endpoint was affected even at the lowest concentration of MO injected, when all other endpoints (survival, hatching rate, presence of curved tail phenotype) did not differ from those of controls. Similar phenotypes have been described in animals carrying mutations that affect the balance systems and/or the swimming behaviour, so-called motility mutants (Ashmore, 1998). The inner ear was found to be affected in the mutants that exhibit the circling behaviour (Nicolson et al., 1998; Whitfield et al., 1996). Thus, the circling swimming behaviour observed in our morphants indicated a disruption of inner ear functions. As the gross morphology of the inner ear in morphants developed normally (data not shown), the circling is most probably due to the inability of ER\( \beta_2 \) morphants to differentiate functional hair cells not only in the neuromasts but also in the inner ear.

This study showed for the first time the role of ER\( \beta_2 \) in the development of the hair cells in the lateral line of zebrafish. Our findings also support different reports documenting the role of estrogens in the inner ear development. ER expression was detected in the inner ear of mouse and rat (Stenberg et al., 1999). Hearing was disrupted in ER\( \beta\)ko mice (Hultcrantz et al., 2006). In humans, a study looking at menopause women revealed that estrogen therapy may slow down hearing loss (Kilicdag et al., 2004). In fish, ER\( \alpha \) has been detected in the inner ear of midshipman (Forlano et al., 2005). Reduced levels of estrogens prevented midshipman females from hearing the reproductive songs of the male (Sisneros et al., 2004). The overall morphology of the hair cells of the inner ear and lateral line is conserved among vertebrates. Moreover, a number of genes required
3.4 Discussion

for hair cell function in the zebrafish has been associated with auditory defects in mice and humans, thus revealing the conservation of function of these genes (Nicolson, 2005). These facts along with the reports documenting the role of estrogens in the inner ear development provide further support for the role of ERβ2 in the development of hair cells in zebrafish, discovered in the present study.

**Notch family members are up-regulation in ERβ2 morphants**

We have performed DNA microarray analyses to investigate further the genetic mechanisms leading to disruption of hair cell differentiation in the neuromasts of ERβ2 morphants. The acquired data showed an up-regulation of two homologous notch genes, *notch1a* and *notch3*. The latter was found to be up-regulated only in the morphants injected with higher concentration of ERβ2 MO. Interestingly, both of the Notch ligands (*deltaA* and *deltaB*) were found to be up-regulated in morphants injected with both MO concentration, which supports the activation of the Notch signalling pathway. The involvement of Delta/Notch signalling in hair cell differentiation has been shown before in both neuromasts and inner ear of zebrafish. Increased Notch expression prevents supporting cells from differentiating into hair cells, while a disruption in this signalling leads to abnormal increase in hair cell numbers. This mechanism has been investigated in the mind bumb (*mib*) mutants, which are characterized by presence of supernumerary hair cells in the PLL neuromasts and a disruption in the Notch signalling pathway (Itoh et al., 2003). In the inner ear it has been shown that the nascent hair cells, expressing Delta protein, the ligand of Notch, inhibit their neighbours from differentiating into hair cells, forcing them to be supporting cells instead (Haddon et al., 1998a). Studies in mice also revealed this mechanism of lateral inhibition that plays a major role in determining the fate of the neuroepithelial cells of the inner ear (Bryant et al., 2002).

The *notch3* gene was found to be up-regulated in the high MO concentration array and was clearly induced in the neuromasts of the low concentration MO-injected embryos’, as shown with ISH (Figure 3.8 A-D). The fact that *notch3* up-regulation has not been found in one of the screens may be related to the consideration that arrays have been performed on RNA extracted from the whole organism, thus masking the occurrence of local induction. Opposite to *notch3*, *notch1a* was found to be up-regulated in both screens but locally its expression was weak and no clear induction could be detected with ISH (Figure 3.8 E-F). These findings may suggest that the main Notch receptor playing a role in cell fate decisions in neuromasts is the Notch3 or/and that these two are collaborating, as has been already shown in other systems in zebrafish, for example in the epidermal...
Estrogen receptor subtype $\beta_2$ is involved in neuromast development

ionocytes (Hsiao et al., 2007) or in the pronephric duct (Ma and Jiang, 2007).

A recent study showed that after ablation of hair cells by neomycin treatment, the supporting cells started to proliferate and later differentiated into new hair cells (Rodbell, 1995). Different members of the Notch signalling pathway ($notch3$, $deltaA$, $atoh1a$) were found to be up-regulated during the time of maximum proliferation of supporting cells and formation of hair cell progenitors, suggesting a regulation via Notch signalling. Although in the present study we did not look directly into proliferation of supporting cells and hair cells renewal after ER$\beta_2$ knock down, the discovery of up-regulated notch expression in the supporting cells of neuromasts in ER$\beta_2$ morphants may suggest that disturbed Notch signalling is involved in the suppression of hair cell differentiation.

Our findings indicated a direct or indirect interaction between transcription factor ER$\beta_2$ and members of Notch signalling pathway, supporting a few other studies reporting on the interaction between Notch pathway players and steroid hormones. Notch activation has been observed in the cells treated with tamoxifen, an ER antagonist (Rizzo et al., 2008). Another study revealed a cross-talk between Notch and androgen pathways in human prostate (Belandia et al., 2005; Belandia and Parker, 2006). In order to get more information about a cross-talk between estrogens and Notch signalling, it would be interesting to see if hair cells would differentiate in ER$\beta_2$ morphants after additional knock down of $notch$ or after injection of ER$\beta_2$ MO in the mib mutant which has a disrupted Delta/Notch signalling.

ER$\beta_2$ role in neurogenesis

The Delta/Notch signalling pathway is also known to be involved in neurogenesis, where the neurogenic genes $delta$ and $notch$ together with the proneural genes, neurogenin ($ngn1$) and neuroD ($nrd$), the two most important transcription factors involved in neurogenesis, establish the nervous system, including the sensory system of the embryos (Bertrand et al., 2002; Sarrazin et al., 2006). Gain- and loss-of-function studies revealed that $ngn1$ activates a generic neuronal program and determines with local signals the particular subtype of neuron that is formed (Bertrand et al., 2002). It is also essential for the development of the sensory system of most of the cranial ganglia, including those innervating the lateral line (Blader et al., 1997). Besides the loss of sensory neurons, $ngn1$ knock down revealed also a strong down-regulation of $nrd$ (Anderson et al., 2002).

Our microarray data provided indications that the overall process of neurogenesis is altered in ER$\beta_2$ morphants. The genes $ngn1$ and $nrd$ were found to be strongly up-
regulated in the microarrays, also confirmed with ISH for ngn1 (Figure 3.7). Interestingly, ngn1 was not only up-regulated in morphants, but also showed an altered expression pattern. It was found to be exclusively expressed in motor neurons along the neural tube instead of localizing in sensory neurons along the lateral line. Thus, the disruption in the development of sensory neurons may explain why the morphants were not reacting to tactile stimuli. It would be interesting to investigate if the pattern of nrd expression is also altered in morphants, as it is expressed in the lateral line neuromasts (Sarrazin et al., 2006) and plays a role in neuronal differentiation together with ngn1 (Bertrand et al., 2002). Further studies should investigate if the neurons that innervate the hair cells of the neuromasts are affected by the lack of ERβ2 or by the up-regulation of the ngn1 and nrd genes itself. We show that ERβ2 function is required for the normal development of the sensory system, which arises from the CNS. In mammals, ERβ plays a predominant role in the CNS, as was shown with the ERβko mice model (Bodo and Rissman, 2006; Gustafsson, 1999). Elucidation of the role of ERβ2 in the development of the CNS was not the goal of this study, however, our data suggest that ERβ2 might play a role in neuronal development in zebrafish.

Conclusions

The present study added to the knowledge on the multiple functions of estrogens in vertebrates. With loss-of-function analysis we have shown that ERβ2 is needed for the hair cell differentiation in the neuromasts, supported the presence of cross-talk between estrogens and Notch signalling pathways and provided indications for the involvement of ERβ2 in the process of neurogenesis during early development of zebrafish. Further research should concentrate on the identification of the exact mechanisms by which ERβ2 mediates these multiple functions and of the specific roles that different receptors play during development.
3 Estrogen receptor subtype $\beta_2$ is involved in neuromast development

Acknowledgements

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3.4 Discussion
CHAPTER 4

ZEBRAFISH (DANIO RERIO)

NEUROMAST: PROMISING BIOLOGICAL ENDPOINT LINKING DEVELOPMENTAL AND TOXICOLOGICAL STUDIES

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Abstract

Aquatic toxicology is facing the challenge to assess the impact of complex mixtures of compounds on diverse biological endpoints. So far, ecotoxicology focuses mainly on apical endpoints such as growth, lethality and reproduction, but does not consider sublethal toxic effects that may indirectly cause ecological effects. One such sublethal effect is toxicant-induced impairment of neurosensory functions which will affect important behavioural traits of exposed organisms. Here, we critically review the mechanosensory lateral line (LL) system of zebrafish as a model to screen for chemical effects on neurosensory function of fish in particular and vertebrates in general. The LL system consists of so-called neuromasts composed of centrally located sensory hair cells and surrounding supporting cells. The function of neuromasts is detection of water movements what is essential for the fish’s ability to detect prey, to escape predator, to socially interact or to show rheotactic behaviour. Recent advances in the study of these organs provided re-
4.1 Introduction

searchers with a broad area of molecular tools for easy and rapid detection of neuromasts dysfunction and/or disturbed development. Further, genes involved in neuromasts differentiation have been identified using auditory/mechanosensory mutants and morphants. A number of environmental toxicants including metals and pharmaceuticals have been shown to affect neuromasts development and/or function. The use of the LL organ for toxicological studies offers the advantage to integrate the available profound knowledge on developmental biology of the neuromasts with the study of chemical toxicity. This combination may provide a powerful tool in environmental risk assessment.

4.1 Introduction

The aquatic resources are severely affected by pollution, since human population keeps growing concomitant with the ever increasing use and release of chemicals into the environment (Schwarzenbach et al., 2006). Ecotoxicology helped to reduce the risk of acute effects caused by the release of high volumes of industrial chemicals. The subtle and chronic effects caused by specifically acting compounds derived for example from personal care products or pharmaceuticals, are not yet adequately addressed by the standard toxicological tests (Segner, 2007; Eggen et al., 2004; Cunningham et al., 2006). These compounds, occurring at low concentration and in mixtures, affect subtle physiological traits in organisms and may directly or indirectly cause long-term adverse ecological effects (Scott and Sloman, 2004).

Examples of these compounds are the pharmaceuticals and the heavy metals that are known to affect the sensory system of humans as well as of fish. When pharmaceuticals are administered to patients, some of their active ingredients may not be completely metabolised (Anderson et al., 2004; Schwab et al., 2005; Kolpin et al., 2002). In a recent study it was described that 50% of the drugs found in the waste water are in the original bioactive form, and 50% are metabolized (Lienert et al., 2007). Since the drugs have been designed to be active, it is to be expected that these compounds and their metabolites will have similar effects in the aquatic environment and may also have toxic effects on non-target organisms. These compounds and their metabolites are only partially removed by actual waste water treatment plants, and hence they are, though at low concentrations, found in the environment. A recently well-studied representative of drugs entering the aquatic environment and having effects on aquatic organisms is the synthetic hormone, 17β-ethinylestradiol, which has been shown to have endocrine disrupting effects in wildlife (Nash et al., 2004; Fenske et al., 2005). In addition, human-related activities, such as industrial and consumer waste, or acidic rain, release heavy metals, which contaminate
streams, lakes, rivers, and groundwater. Metal pollution is a serious concern for aquatic wildlife as these inorganic compounds tend to accumulate in biological organisms.

While for the risk assessment of acute exposures to non-specifically acting chemicals ecotoxicology has developed a valuable set of toxicological tools, the assessment of low-dose, chronic effects of more specifically acting compounds asks to evolve novel, more mechanisms-oriented tools (Eggen and Segner, 2003; Eggen et al., 2004; Hutchinson et al., 2006). More emphasis has to be placed on the toxicant-induced impairment of physiological functions that does not directly lead to lethality but still significantly diminishes the ability of organisms to cope with their environment (Relyea and Diecks, 2008). One such function is the sensory system, which is essential for behaviour, social interactions, prey detection and predator avoidance. Toxicant-induced impairments of sensory functions have therefore an immediate ecological relevance. In fact, it has been shown that a variety of chemicals are able to alter sensory function and structure (Hansen et al., 1999; Bettini et al., 2006; Ottinger et al., 2008). In humans, some pharmaceuticals showed to affect hearing and balance disorders and heavy metals affect the vision, taste, olfaction and auditory functions (Gobba, 2003; Loeffler and Ternes, 2003). These chemicals reach the environment and affects similarly the aquatic organisms (Loeffler and Ternes, 2003). In fish, aminoglycoside antibiotics and heavy metals have been affect the olfactory and mechanosensory systems (Hansen et al., 1999). The latter system is closely related to the hearing system of higher vertebrates. Beside possessing the typical vertebrate inner ears with both hearing and vestibular organs, some fish possess additional structures that contain sensory hair cells, the lateral line (LL) organs (reviewed by Popper (2000)). The LL is composed of rosette-like structures called neuromasts, located on the surface of the animal and readily accessible to analysis. The neuromasts on the head form the so-called anterior LL system (ALL). The posterior LL (PLL) consists of the neuromasts of the trunk and tail. Mature neuromasts are composed of hair cells and supporting cells (Ghyssen and Dambly-Chaudiere, 2007, 2004). The LL hair cells are supposed to gain their mechanotransduction capacity at 3-4 days post hatching (dph) as it is at this time when they become innervated (Raible and Kruse, 2000) and when larval behaviours become consistent with possessing a functional LL (Nicolson et al., 1998). The function of the mechanosensory LL is mainly to allow fish to orient relative to a water current (rheotaxis), to hold a stationary position in a stream, to detect prey, and to avoid predators.

The LL has been particularly well studied in zebrafish, an excellent model organism frequently used in (eco)toxicology (Segner, ress; Hill et al., 2005; Scholz et al., 2008), pharmacological screening (Zon and Peterson, 2005; Goldsmith, 2004) and neurotoxicology (Linney et al., 2004). Zebrafish embryos enable small-scale and high-throughput
analyses. Moreover, besides looking at acute toxicity, fish embryos are also suitable for detecting possible adverse long-term effects. The zebrafish LL is a favourable system to explore gene function and effects of contaminants, given its simplicity, resolution, sensitivity to toxicants and accessibility.

The zebrafish LL analysis offers many advantages, particularly when compared to cell lines studies, where it is difficult to mimic in vivo conditions (Stone et al., 1996; McFadden et al., 2003). Indeed, cell-based assays do not allow assessment of the complex metabolism that causes toxicity in animals (Stone et al., 1996; McFadden et al., 2003). For example, cultured cochlear epithelia lack supporting cells which also play a role in hair cell functions (Ton and Parng, 2005). Neuromasts have been shown to be an excellent system for studying ototoxicity (damage of the ear), as their peripheral sensory neurons are in direct contact with the surrounding water containing pollutants. Another advantage is that hair cells can be easily stained by various fluorescent dyes without complicated histological preparation even in the living larva (Collazo et al., 1994; Seiler and Nicolson, 1999; Hernandez et al., 2006a). The level of hair cell staining can be imaged and quantified using morphometric analysis, and the effects can be assessed for the screening of potential ototoxic pollutants (Ton and Parng, 2005). Moreover, the effects of contaminants can also be assessed on the supporting cells, which are not accessible at all in in vitro studies.

This review summarizes the efforts to elucidate the normal development of neuromasts and its disruption by toxicants that have been reported so far (Figure 4.1). The parallels drawn between blocking LL development through gene knock down or through chemical treatment show that this organ might provide a powerful tool to combine developmental and toxicological analyses. In fact, the more molecular understanding we get of the normal developmental process of neuromasts formation, the better tools will be available to assess toxic effects of pollutants on this sensory system.
4.2 Gene function studies in neuromasts

In order to be able to evaluate the effects of pollutants on the LL system, it is important to understand the molecular basis of its development. In this part of the review, we will focus on research performed in zebrafish that can link genetic expression with function in setting up the LL. Many genes involved in normal development of the sensory LL, have been identified and functionally characterized (Table 4.1). The knowledge in this area increased very fast in recent years mainly because of the availability of several mechanosensory mutants, as well as of a simple reverse genetic technique, the morpholinos (MO) knock down technology. Importantly, the development of the LL can be easily blocked and the consequences of disrupted neuromast development can easily be detected on the physiological and behavioural level, so that molecular changes can be phenotypically anchored. The following paragraphs are structured around the genes that play a role in placode formation, primordium migration, pro-neuromast deposition, hair cell differentiation and hair cell apical surface endocytosis.
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4 Zebrafish (Danio rerio) neuromast: promising biological endpoint

4.2.1 Genes involved in placode formation

Placodes arise from the neural ectoderm in a specific region of the neural plate during early neurogenesis. In response to specific molecular signals, the different cephalic placodes are induced and later developed into diverse sensory organs. For example, the otic placode forms the inner ear and the LL placode forms the LL organs. With the help of different mutants and morphants, the role of involved in LL placode formation has been investigated in zebrafish.

**ngn1 mutants and morphants**

The genes neurogenin (ngn1) and neuroD (nrd) are considered to be the molecular markers for cranial neurogenic placodes in zebrafish. *ngn1* is expressed in neurogenic placodes, including the LL placodes, and in cells within the otic vesicle (Andermann et al., 2002). The otic vesicle generates the sensory hair cells in the inner ear, while the LL placodes give rise to sensory ganglia and to migrating primordia that deposit neuromasts along the head and body (Gompel et al., 2001).

In *ngn1* morphants, the differentiation of all cranial ganglia is blocked, as was shown with specific markers of neuronal cell bodies (anti-HU antibody) and of neurogenic placodes (*nrd*); cranial ganglion neurons were absent and *nrd* expression was down-regulated in neurogenic placodes. The latter observation suggested that *nrd* acts downstream of *ngn1* in the development of LL placodes (Andermann et al., 2002). The morphants were also touch insensitive, suggesting a role of *ngn1* in the formation of trunk sensory neurons. The formation or migration of the LL primordium was not affected in the *ngn1* morphants, as was shown by the presence of eyes absent-1 (*eya1*) expression, a primordium marker, and by the presence of neuromasts at the tip of the tail, as well as differentiated hair cells (Andermann et al., 2002). This study showed that LL neuromasts, derived from migrating LL primordia, can differentiate and persist for several days even in the absence of differentiated LL ganglia. In addition, interactions with the nerve were not necessary for neuromasts differentiation. This suggests that placode-derived neuromast precursors develop in the absence of *ngn1* function, and thus that the two derivatives of the LL placodes, the ganglia and the migrating primordia, are under separate genetic control.

These results have been confirmed by the study of Lopez-Schieer et al. (2004), where the formation of supernumerary neuromasts between neuromast of normal larvae has been shown in *ngn1* morphants and mutants. The presence of these intercalary neuromasts could not be explained by an increase in primordial cells or by higher mitotical activity of primordia. Also at 48 hours post fertilization (hpf) no difference in the number of
4.2 Gene function studies in neuromasts

neuromasts were found, suggesting that the supernumerary neuromasts in *ngn1* mutants do not originate during the initial development of the LL. The extra neuromasts formed were finally attributed to the absence of glial, supported by phenotype comparison with *colourless* mutants that show similar interneuromasts and are characterized by the absence of *sox10* gene coding for a transcription factor associated with the development of nonectomesenchymal derivatives of the neural crest. The absence of *sox10* leads to defective development of peripheral glia but does not affect the development of sensory axons innervating the LL (Lopez-Schier et al., 2004). This research confirmed that *ngn1* gene is not necessary for the migration of the primordium and the initial development of neuromasts, but is needed for the generation of the sensory ganglia from the placode. The intercalary neuromasts phenotype observed in *ngn1* morphant fish is due to the absence of the glial cells that normally accompany the migrating LL nerve. This lack of glia obviously inhibits the assembly of interneuromast cells into neuromasts (Lopez-Schier et al., 2004).

### 4.2.2 Genes involved in primordium migration and pro-neuromast deposition

The PLL ganglion is formed from the differentiated LL placode and contains a large compartment of around 100 cells, the so-called PLL primordium. At 20 hpf the primordium begins to migrate and neuromast deposition occurs. During this migration clusters of cells are deposited, the so-called pro-neuromasts (Sapede et al., 2002). The LL emerged as a favourable system for analysing the genetics of cell migration and of its control.

**sdf1** and **cxcr4** morphants

The CXC chemokine receptor (CXCR4) and its ligand, stromal-derived-factor-1 (SDF1) have been shown to drive the migration of the primordium and to define its stereotyped migration route (David et al., 2002). After knock down of either the ligand or the receptor, the primordium was found to move little or not at all at 32 hpf. At 52 hpf, when the PLL normally comprises a line of 7-8 neuromasts, the *sdf1a* morphants showed no or very few neuromasts along the trunk. However, the neuromasts of the head developed normally. Thus, *sdf1a* is an essential component regulating migration of the PLL primordium. The inactivation of *cxcr4b* at 3 dpf also resulted in major defects in the PLL, while the ALL developed normally (David et al., 2002). The authors concluded that the migration of the PLL primordium is predominantly regulated by the interaction between the SDF1 ligand, which determines the path, with its CXCR4 receptor, that controls the movement. Further
research revealed that also other receptors are involved in the primordium migration. In a recent loss-of-function study it was shown that the CXCR4 is only needed for the cells at the very tip of the tail (Haas and Gilmour, 2006), while in the trailing region CXCR7 seems to be necessary for primordium migration (Valentin et al., 2007).

cadherins morphants and mutants

Cadherins, a type of cell surface molecules, have been shown to play a role in cell adhesion. Different members of the cadherin family have been found in zebrafish neuromast, namely cadherin-2 (cdh2), cadherin-4 (cdh4) and cadherin-23 (cdh23). Cdh2 has been found to be expressed in developing cranial ganglia, in the LL ganglia and in neuromasts. The LL system did not develop normally after knock down of cdh2 (Kerstetter et al., 2004). The morphants had a reduced number of neuromasts on the head, trunk and tail, some neuromasts were located too close to each other. The exact underlying mechanisms of the disrupted processes are not known. Additional data from cdh4 morphants revealed that the cranial and LL ganglia were disorganized, appeared smaller and altered in shape compared to control embryos. Shorter LL nerves and reduced number of neuromasts were also observed, suggesting a disrupted migration of the LL primordium after knock down of cadherins (Wilson et al., 2007). Sputnik mutant showed mechanosensory defects caused by the cdh23 mutation. This study showed a role of cdh23 in the development and signal transduction of the extremity of hair bundles (see section 4.2.4) (Sollner et al., 2004). It needs yet to be verified whether cadherins are important for primordium migration or for hair cell development, or for both processes.

tacstd morphants

The tumour-associated calcium signal transducer gene (tacstd) codes for cell surface glycoproteins and is postulated to have a function in cell adhesion, tumorigenesis and regulation of proliferation in mammalian cells (Went et al., 2004). In zebrafish, the tacstd gene encodes a membrane protein that is homologous to the TACSTD1/2 mammalian proteins (Villablanca et al., 2006). A part of the molecular machinery involved in primordium migration is shared with that of invasive tumor cells. TACSTD expression was found in ALL and PLL neuromasts. At 28 hpf, expression is maintained in the migrating PLL primordium as well as in pro-neuromasts, and later it is observed in the mature neuromasts. Within the pro-neuromasts and mature neuromasts, the tacstd expression pattern appears ring-like, suggesting specific expression in the supporting cells (Villablanca et al., 2006). Comparison of tacstd expression with that of the proneural gene, the atonal ho-
4.2 Gene function studies in neuromasts

mologue \((\text{atoh1a})\), allowed concluding that both are expressed in largely complementary patterns in pro-neuromasts. It was discovered by a loss-of-function analysis that \(\text{tacstd}\) is necessary for neuromast development. A significant reduction in number, or even absence of neuromasts was observed in \(\text{tacstd}\) morphants. As one or two terminal neuromasts at the tip of the tail could still be found in some morphants, the authors concluded that \(\text{tacstd}\) is not involved in primordium migration, but rather plays a role in cell deposition. This is confirmed by the presence of \(\text{tacstd}\) in both ALL and PLL neuromasts, suggesting that \(\text{tacstd}\) is not involved in the control of CXCR4-dependent migration, as CXCR4 drives exclusively the PLL primordium and not the ALL (David et al., 2002).

**\text{met}** morphants

Receptor tyrosine kinase (Met), a high affinity cell surface receptor, and its ligand, hepatocyte growth factor (Hgf), are key regulators of normal cellular processes as well as of development and progression of many cancer types. In zebrafish, Met and Hgf are required for the correct morphogenesis of the hypaxial muscles in which \(\text{met}\) transcripts have been detected (Haines et al., 2004). Met signalling showed to be needed for pro-neuromast deposition by the PLL primordium. Met is present during the entire period of primordia migration, but it is not expressed within deposited pro-neuromasts or neuromast clusters. \(\text{Met}\) morphants showed a reduction in the number of deposited neuromast clusters stained with a fluorescent dye. The number of hair cells within the deposited neuromast was also reduced. With the supporting cell marker gene, \(\text{follistatin}\) a lack of supporting cells was also revealed. The authors identified that \(\text{met}\) plays a role in neuromast deposition, but not in the correct migration of the PLL primordia along the body axis, as shown with a not affected primordia marker gene, \(\text{prox1}\) (Haines et al., 2004).

Altogether, the primordium migration is determined by the interaction between the SDF1, CXCR4 and CXCR7. Two molecule types essential for cell adhesion have also been shown to be important for neuromast development: the cadherins, which are suspected to have a role in cell migration, and the TACSTD which together with Met is needed for neuromast deposition.

4.2.3 Genes involved in hair cell differentiation

After cell deposition, the neuromast maturation is initiated. The cells are differentiating and a rosette shape structure is formed with the supporting cells in the periphery. In the center, the cells differentiate into hair cells with stereocilia and kinocilium. The mind bumb mutants, together with other mutants and morphants, helped to decipher the
process of hair cell differentiation.

**mind bomb (mib) mutants**

The *mib* mutant has been discovered by searching for neurogenic phenotypes in the Boston zebrafish mutagenesis screen (Haddon et al., 1998a). The zebrafish *mib* mutants carry a mutation in the *mib* gene, which encodes a RING E3 ligase required for Notch activation (Itoh et al., 2003; Whitfield, 2005). A failure of lateral inhibition mediated by Notch signalling is observed in these mutants (Itoh and Chitnis, 2001; Itoh et al., 2003; Haddon et al., 1998b). The phenotype of this mutant, characterized by supernumerary hair cells, (Jiang et al., 1996; Schier et al., 1996), was used to analyse the different roles of proneural genes in hair cell differentiation.

Notch/Delta signalling is known to play a role in cell fate decision in hair cells of the mammalian inner ear (Hawkins et al., 2007). A similar mechanism is involved in hair cell differentiation in zebrafish neuromasts. The normal expression patterns of *notch2*, *deltaA*, *deltaB*, and *atoh1a* were analysed and compared with those in the *mib* mutant (Itoh and Chitnis, 2001). The expression of *notch3* in the *mib* mutants was down-regulated in the primordium of the LL, suggesting that it is excluded from selected hair cells in the maturing neuromasts. The three other proneural genes showed to be up-regulated in the hair cells of the neuromast primordium. The *atoh1a* expression was not restricted to specific cells, thus causing the formation of too many hair cells at the cost of supporting cells. This correlates with a corresponding increase in the number of cells expressing *deltaA* and *deltaB* (Itoh and Chitnis, 2001). Furthermore, a recent study showed a role of the different members of the Notch signalling pathway in the hair cell regeneration process. Hair cells were able to regenerate after neomycin damage. The renewal of hair cells was accompanied by an increase in supporting cell proliferation and by a *notch3*, *deltaA* and *atoh1a* up-regulation (Ma et al., 2008).

In addition to the *atoh1a* up-regulation, *nrd* also showed to be up-regulated in hair cell precursors of the *mib* mutant. The loss-of-function analysis of both genes revealed that *atoh1a* and *nrd* are essential for hair cell development, but not for other cell types in the neuromasts (Sarrazin et al., 2006). The phenotype produced by the loss of *atoh1a* was rescued by injection of *nrd* mRNA. This suggested that in the hair cell context *nrd* is regulated by *atoh1a*, whereas in sensory neurons *nrd* is regulated by *ngn1*.

Also, *six1* expression was investigated in the *mib* mutant (Bessarab et al., 2004). The *six* gene family is known to be involved in morphogenesis, organogenesis, and cell differentiation. The expression of *six1* at 12 hpf was observed in the region that later gives rise to the otic vesicle as well as ALL and PLL placodes. At 24 hpf, *six1* was mostly expressed
4.2 Gene function studies in neuromasts

in the otic placodes, LL placode, vestibular ganglia and somites. In neuromasts of the midbody LL, the *six1* expression was detected first at 48 hpf in wildtype, reaching its peak at 72 hpf with stronger staining at the basal region of the neuromast, where bodies of hair cells are localized. In the *mib* mutant, *six1* expression was elevated at 48 hpf in the neuromasts. In addition, the excessive and premature production of hair cells was seen in the sensory patches of the *mib* inner ear (Haddon et al., 1998a). These results suggested that, during sensory cells differentiation in the inner ear and LL, *six1* is regulated by Notch pathway (Bessarab et al., 2004).

dog-eared mutants (-eya1)

The zebrafish mutant dog-eared is characterized by abnormal morphology of the inner ear and LL sensory system (Whitfield et al., 1996). The dog-eared embryos are less responsive to vibrational stimuli, fail to maintain balance when swimming, and may circle when disturbed, a characteristic of fish with vestibular defects (Nicolson et al., 1998). In the developing otic vesicle and migrating primodium of the PLL, the homozygous dog-eared embryos showed cell death in abnormal places and in increased levels. The dog-eared locus encodes the eya1 gene. It is known that eya1 is required for cell differentiation and survival in the fly eye (Bonini et al., 1993). The eya1 gene is expressed during embryogenesis in cells of the anterior pituitary, olfactory, otic, and LL placodes, as well as in the somites, branchial arches and pectoral fins of zebrafish (Sahly et al., 1999). A recent study showed that eya1 is required for survival of cells in the otic vesicle, including those cells that will differentiate into hair cells (Kozlowski et al., 2005). Comparison of the dog-eared mutants with the eya1 MO injected embryos showed that both phenotypes are similar, suggesting that eya1 is required for cell survival in the otic vesicle, including the cells that will differentiate into hair cells.

tmie morphants

In mice and humans, transmembrane inner ear gene (*tmie*) mutation causes vestibular dysfunction and profound hearing loss. In mice, *tmie* is expressed in the inner ear, brain, liver, kidney and lung. In zebrafish, the expression of *tmie* was found in the brain and the inner ear (Shen et al., 2008). After knocking down *tmie* expression, the morphants showed abnormalities in swimming behaviour. Most morphants swam in circles and exhibited altered swimming after touch stimuli or dish swirling even at 6 days post fertilization (dpf). The morphants also lost their balance control. Further investigation on the development of the inner ear and LL system using a fluorescent live staining revealed that morphants
had fewer active hair cells than control fish (Shen et al., 2008). The expression of *excr4b*, a primordium marker, was used to show that the lack of hair cell activity is not a result of delayed migration of the LL primordium. The expression of *atoh1a* showed that the hair cell differentiation occurs soon after the deposition of pro-neuromasts, indicating that the early differentiation of the hair cells was not affected by *tmie* knock down. However, the delay in the maturation and/or the activity of the hair cells suggests that *tmie* is participating in maturation, function and possibly maintenance of the hair cells in the inner ear and LL organs.

**ru848 mutants (-*chm*)**

After a mutagenic screen performed to find proteins important for hair cell differentiation, larvae showing inner ear and LL organ development and function defects, causing acoustic and balance disorders, were selected, and the phenotypes were further analysed. It was discovered that the mutant *ru848* lacks 90% of the hair cells at 5 dpf (Starr et al., 2004). The authors identified the mutated gene to be the choroideremia (*chm*) gene and confirmed its role in hair cell development using the knock down technique. This gene encodes the Rab escort protein 1, known to play a role in many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, as well as membrane fusion. Using live staining and scanning electron microscopy (SEM), they could identify that in *chm* morphants the periderm cells appeared relatively normal, but in the center of the neuromast less kinocilia were detected and were much shorter than those seen in the wildtype (Starr et al., 2004). The retina was also affected by this mutation. It is known that the same mutation in humans causes choroideremia, a disease marked by slow-onset degeneration of photoreceptors and retinal pigment epithelial cells, suggesting that this mutant, besides being a model for hair cell analysis may also be a model for degenerative disease in human retina.

**hi472 mutants (-*zVIPL*)**

A retroviral insertional mutagenesis screen showed that *hi472* mutation, caused by a retroviral insertion into the vesicular integral protein-like gene (*zVIPL*), resulted in a reduction of mechanosensitivity, indicated by a loss of escape behaviour (Chong et al., 2008). The *hi472* mutant suffers from a severe loss of hair cells in the neuromasts, as well as a reduction in supporting cells. The *zVIPL* mutation affected the Delta-Notch signalling, leading to an increase of notch expression. The phenotype could be partially rescued by treatment with an inhibitor of Notch signalling. Therefore, *zVIPL* is a nec-
4.2 Gene function studies in neuromasts

Essary component of Delta-Notch signalling during neuromast development in the LL of zebrafish.

\textbf{erβ2 morphants}

The presence of hormone receptors in the sensory hair cells has been examined in our laboratory. Recently, estrogen receptor β2 (erβ2) mRNA has been shown to be expressed in supporting and hair cells of zebrafish LL (Tingaud-Sequeira et al., 2004). After knock down, the larvae showed a circling swimming behaviour, typical for mechanosensory mutants. Staining with a vital dye showed that the neuromasts of the morphants lacked functional hair cells. Using cellular marker for supporting cells (claudinb and keratin15), a normal migration of the primordium and deposition of the supporting cells was observed. However, after injecting the erβ2 MO in ET4 transgenic fish expressing green fluorescent protein (GFP) in the hair cells (Parinov et al., 2004), a lack of hair cell development was observed, suggesting a role of erβ2 in hair cell differentiation. Using microarrays, an up-regulation of the notch1a and notch3 gene was measured, also confirmed by ISH in the supporting cells of the neuromast (Froehlicher et al. (2008) in prep). These data suggest an interaction between the estrogen and the Notch signalling pathways during hair cell differentiation in the LL.

In conclusion, besides the proneural genes such as deltaA, deltaB, atoh1a, nrd, six1, several other genes revealed to be essential for hair cell development in the LL of zebrafish. The gene eya1 showed to be essential for hair cell survival, tnie was suggested to be involved in hair cell maturation, function or maintenance, chm showed to be needed for the presence of normal kinocilia, zVIPL for the supporting and hair cell development and erβ2 for hair cell differentiation.

4.2.4 Genes involved in hair cell apical surface endocytosis

Sensory hair cells need a fast rate of synaptic release in order to transmit the mechanosensory signals to the neuronal system. This is ensured by the surface of hair cells that is pitted with many invaginations of the apical membrane. This membrane is thought to have a high turnover rate, with a permanent replacement of old proteins by freshly synthesised proteins. The release and uptake of synaptic transmitter and the renewal of membrane proteins, such as ion channel proteins, are guaranteed by the mechanism of endocytosis. Different pathways are known to play a role during endocytosis, including clathrin-dependent and caveolin-dependent pathways.
**cav1α morphants**

Caveolins are integral membrane proteins contained in specialized plasma membrane microdomains called caveolae. They are known to be expressed in endothelial cells and adipocytes and to play a role in endocytosis and oncogenesis (Cohen et al., 2003). In zebrafish, *caveolin-1 (cav1α)* expression has been studied and its expression revealed to be absent from the migrating primodium but was detected in the mature neuromasts from 48 hpf. The expression pattern in the ALL and PLL neuromasts exhibits a ring-like shape, which would suggest that it is localized in the supporting cells (Nixon et al., 2007). The neuromast maturation was disrupted after knock down of *cav1α*. A decrease in the number of functional hair cells in the neuromasts of the PLL was observed at 72 hpf using a fluorescent dye. However, the migration of the primordium seemed not to be altered, as by 72 hpf, some neuromasts were observed in the tail but not along the body of the fish. This study showed a vital role of *cav1α* in neuromast development (Nixon et al., 2007). The exact function of *cav1α* in neuromast development is, however, not completely clear. Further investigations are needed to determine its role in either maturation or apical surface endocytosis of zebrafish hair cells.

**ru920 mutants (-myo6b)**

Myosin VI has been found to play a role in hair cell mechanotransduction (Kappler et al., 2004). The authors were using the *ru920* mutant that showed an inability to respond to vibrational and touch stimuli. The mutants circled back and swam upside down. Further analysis showed that, although the LL hair cells in the mutants developed normally, they displayed diminished ability to internalize a specific dye (DiAsp), which enters through mechanoelectrical transduction channels. This suggested that the electrical response failed in the hair cells of the mutants. The mutation was localized in the *myo6b* gene, which is one of the two zebrafish orthologs of the human gene *myosin VI*, and the phenotype was confirmed by *myo6b* knock down. The authors speculated that the lack of Myo6b might disrupt the removal of membrane from the apical surface by clathrin-mediated endocytosis and thereby cause stereociliary fusion.

**mariner, sputnik, orbiter, mercury, gemini, astronaut, cosmonaut and skylab mutants**

All these auditory/vestibular mechanosensory mutants were identified in large-scale mutagenesis screen. They display defects in balance and acoustic startle reflexes owing to peripheral defects in the auditory/vestibular system (Nicolson et al., 1998). When these
4.2 Gene function studies in neuromasts

mutants were tested for their ability to internalize a marker of endocytosis, FM1-43, the majority of them showed a defect in apical endocytosis.

The mariner and sputnik mutants have splayed hair cell bundles and reduced or absent extracellular receptor potentials. The mariner mutant showed to be defective in myosin VIIA, which is expressed in the sensory hair cells (Ernest et al., 2000). This mutation correlates with the shaker-1 mice mutants, defective in the same gene, which are deaf. The zebralsh fish mariner mutants are, therefore, a powerful model for mammalian hereditary deafness. The defect of sputnik is characterized by missing the link between the hair cells extremities, called tip link (Sollner et al., 2004). As mentioned before, it is caused by mutations in the cdh23 gene. The Cdh23 protein is concentrated near the tips of hair bundles. The absence of tip links in sputnik larvae showed that the mechanotransduction but not the hair bundle integrity was affected. Cdh23 is therefore an essential tip link component required for hair-cell mechanotransduction.

Three mutants, orbiter, mercury and gemini, have normal hair cell morphology, but reduced or absent extracellular receptor potentials. Mutants from the third group, astronaut and cosmonaut, also appear to have normal hair cell morphology, and also have conserved their ability to generate extracellular receptor potentials, however, they are partially or completely vibration insensitive. Loss of behavioural responses in these mutants presumably involves defects in later aspects of signal transduction, such as synaptic transmission. A different defect, namely degeneration of sensory hair cells have been shown in the skylab mutant (Nicolson et al., 1998).

When the mariner, sputnik, orbiter, mercury, and skylab mutants were treated with streptomycin, their reduced endocytosis ability was revealed, as they were protected from the cytotoxic effect of this aminoglycoside antibiotic. The effects of similar ototoxic compounds are further discussed in the next section of this review.

As described above, the development of the LL organs occurs in subsequent steps in which various genes are involved. Because of this already substantially combined molecular-physiological insight in the normal developmental process of neuromasts, it is possible to perform mechanistic toxicological studies on the impact of pollutants on disturbed LL development. Different modes of toxic action that occur during the different developmental processes can be studied in greater detail. In the next section, the toxicological studies, that have been reported so far, are reviewed and classified by their biological effects on the neuromasts.
4.3 Neuromast disruption after exposure to toxicants

Toxicological studies nowadays face the challenge to assess subtle and chronic effects caused by a mixture of chemicals present at low concentrations. The subtle effects of environmental compounds on fish’s nervous or sensory system and behaviour have been difficult to assess in the aquatic environment, mainly because the measurement of these endpoints are complex. For this reason, there is an urgent need to develop tools for detecting sensory toxicity. One of such tool could be the sensory LL system of fish. Proper development and function of neurosensory systems such as the LL neuromasts is essential for the ability of organisms to cope with their environment. Therefore, the LL system may serve as experimental screen for chemicals potentially interfering with neurosensory/auditory development and/or function. The molecular mechanisms of the LL development are fairly well studied and sufficient knowhow is available on the analysis of physiological endpoints.

In particular, the hair cells of zebrafish have been shown to be sensitive to the action of drugs and heavy metals. For instance, for aminoglycoside antibiotics, such as gentamicin, intensively used clinically because of its broad spectrum of antibacterial actions, it has been found that some patients suffer from severe hearing problems (Eggen et al., 2004; Forge and Schacht, 2000). Experimental studies using zebra fish could demonstrate that aminoglycoside antibiotics affect the LL system (Owens et al., 2008). In addition, heavy metals were shown to affect the sensory system in humans and orientation and olfactory system in fish (Hansen et al., 1999; Gobba, 2003). Similar to the antibiotics, heavy metals affect the hair cell development of zebrafish. The following section discusses the reports classified by the type of effects that have been observed, i.e. hair cell loss, the potential of hair cell to regenerate and the effects on behaviour related to LL dysfunction after exposure to toxicants.

4.3.1 The effects of toxicants on hair cell disruption

The overall list of toxicants that are known so far to affect the hair cells in zebrafish is presented in Table 4.2 and described in the following paragraphs.

Pharmaceuticals

Aminoglycoside antibiotics

This class of antibiotics is the best documented type of antibiotics known to cause hearing loss in vertebrates. Aminoglycosides are produced by different strains of soil actino-
4.3 Neuromast disruption after exposure to toxicants

mycetes. All natural and semi-synthetic aminoglycosides share a similar structure consisting of several, usually three, rings. The hallmark of aminoglycosides, which chemically might better be termed aminocyclitols, is the presence of amino groups attached to the various rings of the structure. These amino groups and the additional hydroxyl groups convey the major chemical properties, namely high water solubility and a basic character (Forge and Schacht, 2000). The structural features determining ototoxicity remain unknown.

A number of studies showed that aminoglycosides that affect zebrafish hair cells, also affect human inner ear (Harris et al., 2003; Murakami et al., 2003; Ton and Parng, 2005; Chiu et al., 2008; Owens et al., 2008). In all of these investigations, dose-dependent effects on hair cell loss in fish treated with neomycin were observed. Also gentamicin and streptomycin induced hair cell loss (Ton and Parng, 2005; Seiler and Nicolson, 1999). These studies usually assessed the hair cells with fluorescent live staining and in some cases the absence of hair cells were confirmed by transmission electron microscopy (TEM) and SEM (Murakami et al., 2003; Harris et al., 2003). Similarity of the action of these compounds in fish with the ototoxic effects reported in humans suggests that zebrafish LL may be a suitable screen not only for fish but also for other vertebrates.

There are some reports showing developmental differences in sensitivity to aminoglycoside induced hair cell death. In mammals and birds the auditory hair cells become susceptible to the drugs as soon they begin to function as mechanosensory receptors (Friedmann and Bird, 1961; Raphael et al., 1983). Similarly, there is a period of insensitivity to aminoglycosides in zebrafish LL. It is hypothesized that hair cells are at several stages of development within any given neuromast at the time of treatment (Harris et al., 2003; Murakami et al., 2003). It has been analysed whether there is a link between developmental insensitivity to aminoglycosides and mechanotransduction-dependent activity of hair cells (Santos et al., 2006). There were no age-dependent differences in the uptake of a mechanotransduction indicator, the fluorescent dye FM1-43, during the time when stage-dependent differences in susceptibility to neomycin were observed. This phenomenon is also not due to differences in overall maturation of the larvae. Rather, it has been shown that the difference in maturation of individual hair cells, independent of mechanotransduction activity, is determinant. Further research is needed to completely elucidate the basis of immature hair cell resistance to aminoglycoside treatment, for which the specific cell markers presented in the previous section could be used.
### Table 4.2: Compilation of studies detecting neuromast disruption in 4-5 dpf zebrafish (*Danio rerio*) upon exposure to various compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>time of exposure</th>
<th>Measured endpoint*</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aminoglycoside antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>neomycin</td>
<td>10 µM</td>
<td>24h</td>
<td>E</td>
<td>DASPEI</td>
<td>Ton and Parng (2005)</td>
</tr>
<tr>
<td></td>
<td>50, 100, 150, 200, 400 µM</td>
<td>1h</td>
<td>D</td>
<td>DASPEI TEM</td>
<td>Murakami et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>10, 50, 100, 125, 250, 300, 500 µM</td>
<td>1h</td>
<td>D</td>
<td>DASPEI SEM</td>
<td>Harris et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>1.5h</td>
<td>C</td>
<td>BrdU, IHC anti-PH3, acridine orange</td>
<td>Williams and Holder (2000)</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>1h</td>
<td>F</td>
<td>YO-PRO-1, FM1-43FX</td>
<td>Chiu et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>25, 50, 75, 100, 200, 400 µM</td>
<td>30min</td>
<td>F</td>
<td>YO-PRO-1, DASPEI, FM1-43</td>
<td>Owens et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>25, 50, 100, 200, 400 µM</td>
<td>1h</td>
<td>F</td>
<td>FM1-43, TO-PRO-3, YO-PRO-1</td>
<td>Santos et al. (2006)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>5 µM</td>
<td>24h</td>
<td>E</td>
<td>DASPEI</td>
<td>Ton and Parng (2005)</td>
</tr>
<tr>
<td><strong>anti-cancer drugs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cisplatin</td>
<td>50 µM</td>
<td>24h</td>
<td>E</td>
<td>DASPEI</td>
<td>Ton and Parng (2005)</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>1h</td>
<td>F</td>
<td>YO-PRO-1, FM1-43FX</td>
<td>Chiu et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>250, 500, 750, 1000, 1500 µM</td>
<td>4h</td>
<td>F</td>
<td>FM1-43FX</td>
<td>Ou et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>500 µM</td>
<td>4h</td>
<td>F</td>
<td>DASPEI</td>
<td>Owens et al. (2008)</td>
</tr>
<tr>
<td>vinblastine sulfate</td>
<td>100 µM</td>
<td>24h</td>
<td>E</td>
<td>DASPEI</td>
<td>Ton and Parng (2005)</td>
</tr>
<tr>
<td><strong>antiprotozoal: quinine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µM</td>
<td>24h</td>
<td>E</td>
<td>DASPEI</td>
<td>Ton and Parng (2005)</td>
</tr>
<tr>
<td><strong>estrogen: Estradiol valerate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>1h</td>
<td>F</td>
<td>YO-PRO-1, FM1-43FX</td>
<td>Chiu et al. (2008)</td>
</tr>
<tr>
<td><strong>heavy metals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cadmium</td>
<td>0.2, 5, 125 µM</td>
<td>3h</td>
<td>I</td>
<td>ISH hsp70, hsp70/eGFP</td>
<td>Blechinger et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>120h</td>
<td>I</td>
<td>ISH mt</td>
<td>Chen et al. (2007)</td>
</tr>
<tr>
<td>copper</td>
<td>1, 10, 50, 250 µM</td>
<td>2h</td>
<td>B</td>
<td>DiAsp, acridine orange</td>
<td>Hernandez et al. (2006a)</td>
</tr>
<tr>
<td></td>
<td>1, 10 µM</td>
<td>2h</td>
<td>F</td>
<td>ET4, ET20 transgenic line, FM1-43,</td>
<td>Hernandez et al. (2006b)</td>
</tr>
<tr>
<td></td>
<td>68, 244 µM</td>
<td>120h</td>
<td>A</td>
<td>ISH atoh1a, eya1, cldb, IHC anti-Cldb</td>
<td>Johnson et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>5-65 µM</td>
<td>5h</td>
<td>H</td>
<td>DASPEI, SEM</td>
<td>Linbo et al. (2006)</td>
</tr>
<tr>
<td>zinc</td>
<td>50, 250 µM</td>
<td>2h</td>
<td>B</td>
<td>DiAsp, acridine orange</td>
<td>Hernandez et al. (2006a)</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>120h</td>
<td>I</td>
<td>ISH mt</td>
<td>Chen et al. (2007)</td>
</tr>
<tr>
<td>iron</td>
<td>50 µM</td>
<td>2h</td>
<td>B</td>
<td>DiAsp</td>
<td>Hernandez et al. (2006a)</td>
</tr>
<tr>
<td>silver</td>
<td>1, 50, 250 µM</td>
<td>2h</td>
<td>B</td>
<td>DiAsp</td>
<td>Hernandez et al. (2006a)</td>
</tr>
</tbody>
</table>

* Measured endpoints the numbers correspond to: A) % of visible neuromast. B) number of ALL and PLL neuromast. C) number of central cells and peripheral cells. D) % of normal or reduced stained neuromast. E) average of fluorescent intensity in 10 larvae. F) % of survival hair cells in 4 neuromast except for Hernandez et al. (2006b), where two neuromasts were measured. G) mean number of hair cells. H) mean number of hair cell per neuromast. I) imaging of reduced fluorescent dye.
4.3 Neuromast disruption after exposure to toxicants

Other pharmaceuticals affecting the hair cells

Besides aminoglycosides, diverse other medicines have been shown to cause ototoxicity in humans, including the antimalaria drug, quinine, or the well-known aspirin. For a complete list, see the review of Forge and Schacht (2000). Interestingly, cisplatin and vinblastin sulfate, anti-cancer drugs, as well as quinine, were also shown to cause hair cell loss in zebrafish LL neuromasts (Ou et al., 2007; Ton and Parng, 2005; Owens et al., 2008). The authors showed that cisplatin-induced hair cell death occurs in a dose-dependent fashion in the zebrafish LL, in a way comparable to that in mammals.

In a recent study, potential neurotoxic chemicals were screened, and a range of chemicals affecting hair cells has been discovered in zebrafish (Chiu et al., 2008). The authors screened for hair cell toxicity in the LL of zebrafish embryos at 5 dpf, using the library of 1040 Food and Drug Association (FDA)-approved drugs and bioactive compounds (Chiu et al., 2008). They discovered some already known ototoxic drugs to have a significant dose-response effect on the hair cells of the LL in zebrafish. Some compounds with unknown ototoxic effects in humans, including pentamidine, spermadine and vincamin, showed to affect the LL hair cells of zebrafish. Pentamidine and propantheline were further analysed in mammalian cell culture. Mature mouse utricle explants were exposed to these two potential candidate ototoxins, which indeed showed to negatively affect mammalian hair cells. However, no significant hair cell loss was detected after treatment with cisplatin, which has been previously shown to cause hair cell loss in zebrafish (Ou et al., 2007).

Surprisingly, estrogen (estradiol valerate) has been identified in this study as also being potentially ototoxic with respect to hair cells. This contradicts with previous studies where estrogens were shown to have favourable vascular effects protective of hearing (Kilicdag et al., 2004; Hultcrantz et al., 2006). In addition, the ERβ2 knock down caused hair cell loss (see section 4.2.3). These findings need further investigation, as it is most probable that hormone action depends on concentration and therefore contradictory effects may be produced. The knock down of other ER subtypes could be of great interest to investigate the impact of natural and synthetic estrogens on hair cell development in fish.

Screening for hair cell toxicity in the LL of zebrafish could help to decipher the adverse effects of many drugs or environmental compounds. Currently, no test for ototoxicity is included in drug evaluation protocols. Consequently, new drugs are completing clinical trials and reaching the public with no knowledge on ototoxic potential. Moreover, there is a growing environmental concern, as drugs can accumulate in the aquatic environment. Zebrafish could, therefore, be a model organism for enhancing drug tests or to assess the toxicity of the pharmaceuticals and their metabolites already present in the environment.
Using the different mutants and morphants available to trace specific developmental processes, a more detailed analysis of the chemical impacts would be possible.

**Heavy metals**

Among toxic metals, cadmium has been associated with olfactory and sensory dysfunctions in humans as well as in zebrafish (Gobba, 2003; Blechinger et al., 2007). Using transgenic zebrafish strain that expresses the enhanced GFP (eGFP) under control of the *hsp70* gene promoter (*hsp70/eGFP*), the specific cell types that were affected after cadmium exposure could be identified, as *hsp70* gene is known to be up-regulated in cells exposed to toxic metals. The cells that were centrally located in the neuromasts and possessed the typical shape of the mechanosensory hair cells, expressed higher levels of *hsp70* mRNA and consequently the *hsp70/eGFP* reporter gene. The sensory cells of the LL showed to be more sensitive to cadmium than the olfactory system. The authors suggested that cadmium might accumulate in the hair cells, as they showed *hsp70* accumulation and are in direct contact with the chemicals in the water (Blechinger et al., 2007). This study provides us with a rapid marker of cadmium exposure for early detection of sensory cells disruption in LL at concentrations below those that cause rapid and widespread cell death. This feature, allowing to test toxicity below the acute concentration, makes zebrafish LL very attractive to risk assessment.

Other metals also showed to affect the LL organs. A disruption in the neuromasts of zebrafish has been revealed after zinc exposure using a metallothionein (*mt*) ISH probe (Chen et al., 2007). Copper has been shown to affect mainly the olfactory system of the aquatic life. Studies using zebrafish showed that copper also affects the mechanosensory system (Hernandez et al., 2006a; Linbo et al., 2006; Johnson et al., 2007). Even if copper exposure at low concentrations produced no significant effects on growth, morphology or survival, it reduced the number of functional neuromasts. This had the implication such that the fish exposed to copper had a reduced ability to orientate in current, which seriously compromised survival. Also iron and silver revealed to affect hair cell development in fish (Hernandez et al., 2006a; Chen et al., 2007). Altogether, these studies indicate that the heavy metals can cause profound effects on mechanosensory cells of the zebrafish LL, further supporting the suitability of neuromasts assessment for evaluating aquatic pollutants.
4.3 Neuromast disruption after exposure to toxicants

**Mechanisms of hair cell disruption**

Mechanisms of hair cells disruption appear to involve the formation of free radicals and the apoptotic cell death pathways (Forge and Schacht, 2000). It seems that the hair cell death is a consequence of the free radical formation known to create a chain reaction and cause oxidative stress. The typical morphological features of apoptosis have been observed in hair cells exposed to aminoglycosides (Forge, 1985). The aminoglycosides form a complex with iron and activate molecular oxygen, which in turn is reduced to superoxide by an electron donor. As a result, free radicals are formed. One consequence of free radicals production in the cells is the calcium influx disruption, which may result in blocking of cation channels located at the apices of the stereocilia of neuromasts hair cells in the fish LL system (Hudspeth, 1989; Kroese et al., 1989; Forge and Schacht, 2000). This biochemical alteration leads to hair cell loss in zebrafish exposed to ototoxicants. Free-radical production is, therefore, the most probable reason of ototoxicity. This mechanism is supported by the finding that transgenic mice overexpressing the antioxidant enzyme superoxide dismutase are protected from aminoglycoside-induced hearing loss (Sha and Schacht, 1997).

The mechanism by which metals could affect the LL organs is also not completely elucidated. One theory is that cadmium and copper, as well as cobalt and mercury, interfere with the calcium uptake in the hair cells, as it has been shown with the aminoglycosides (Karlsen and Sand, 1987; Liang et al., 2003; Griesinger et al., 2002). The metal ion blocks calcium channels located on the surface of the hair cells by competing with calcium cations at stereocilia level. This disrupts the ion flux and causes hair cell dysfunction (Karlsen and Sand, 1987). Cadmium has been already shown to be a calcium antagonist at the level of the gills (Verbost et al., 1987, 1988). So, as the calcium ions play a preponderant role in signal transduction mechanisms in neuromast hair cells in the fish LL system (Hudspeth and Corey, 1977; Jorgensen, 1984), cadmium ions might affect mechanoreception and thereby alter the behaviour of fish exposed to them.

Even if the mechanism of toxicity of aminoglycosides and heavy metals is proposed, the number of tools available for study of zebrafish neuromasts toxicity could help to decipher further mechanisms by which toxicants could affect the neurosensory system of fish.

**4.3.2 Hair cell regeneration after toxicant exposure**

Some of the experiments described above analysed also the hair cell recovery. The first study revealed that there are two kinds of cell populations, one dying, and the other pro-
liferating (Williams and Holder, 2000). The authors suggested that the dying population of cells are hair cells and the proliferating cells are the periphery cells, called supporting cells. The supporting cells are thought to give rise to hair cells following hair cell death, creating a constant turnover in neuromasts, as already shown in Balak et al. (1990). The recovery of damaged LL was further analysed in the following studies. Zebrafish larvae were checked for number of hair cells 4 and 12 hours after treatment with neomycin at low concentrations and it was found that after 12 hours of recovery more hair cells were stained (Murakami et al., 2003). The authors delivered explanation related to the fact that supporting cells are continuously adding new hair cells to their neuromasts. Since immature hair cells may not be as susceptible to aminoglycoside-induced death, the cells displaying DASPEI labelling at 12 hours that were not labelled at 4 hours may be those that differentiated over this period (Murakami et al., 2003). Harris (2003) also proposed that the youngest hair cells within a given neuromast might survive drug treatment. After neomycin treatment and after 24-48 hours of recovery, hair cells also regenerated in this study (Harris et al., 2003).

Hernandez and co-workers (2006) identified the doses and times of copper exposure that cause reversible and irreversible damage to the zebrafish LL hair cells. As already shown with ototoxic drugs, zebrafish sensory hair cells are able to regenerate. The toxicants, beside inducing hair cell death, also stimulate proliferation among supporting cells in neuromasts, which then over time leads to the generation of new ciliated receptor cells (Williams and Holder, 2000). Hernandez (2006) observed that a regeneration potential was dose-dependent and was different between the ALL and the PLL. Authors suggested that high doses of the metal are able to destroy both the hair cells and most of the cells that have the capacity to generate new hair cells in the PLL. In contrast, in the ALL, even after high doses, abundant numbers of proliferating cells were detected. One explanation could be that ALL neuromasts are embedded in pits or canals in the head (Webb and Shirey, 2003), possibly offering more protection from external toxicants (Hernandez et al., 2006a). In further investigation, transgenic fish that express GFP in different cell types in the LL system were treated with copper (Hernandez et al., 2006b). ET4 transgenic fish have fluorescent hair cells, and ET20 are positive in their supporting cells. After exposure to copper, the hair cells, but not the supporting cells, that were able to give rise to new hair cells, showed to be affected.

The expression of proneural genes, like atoh1a and eya1, required for cell survival, was also lost after copper treatment. Both genes reappeared during recovery, first eya1 and then atoh1a. This study could also show that sox2 and Sox protein are expressed in the supporting cells. This was shown using the ET4 and ET20 transgenic lines, where
4.3 Neuromast disruption after exposure to toxicants

Sox2 expression was not colocalized with the fluorescence of ET4 hair cells, but partially colocalized with the fluorescent supporting cells in the ET20 (Hernandez et al., 2006b). The authors hypothesized that the supporting cells expressing Sox2 could give rise to new hair cells after damage and support the regeneration in zebrafish neuromasts. This assumption was verified by detecting Sox2 expression after copper treatment. At all concentrations, Sox2 expression was detected, which supports the idea that supporting cells survive the treatment and then serve as hair cell precursors.

A very recent study showed that hair cells can regenerate after neomycin treatment from a transient increase in supporting cell proliferation which is accompanied with an up-regulation of notch3, deltaA and atoh1a (Ma et al., 2008). It could be shown that Notch signalling limits the number of hair cells produced during regeneration by regulating supporting cell proliferation.

4.3.3 Effects of toxicants on mechanosensory behaviour

Changed behaviour is a sensitive target of environmental pollutants. Further, it may serve as a link between physiological and ecological processes (reviewed by Scott and Sloman (2004)). Behavioural endpoints may indicate subacute effects of chemicals, like the effects of toxicants on the sensory system. In order to know, whether the cellular effects on the LL have a toxicological relevance, the studies dealing with behavioural changes related to a LL dysfunction are presented in this section and in the Table 4.3.

In the sea bass (*Dicentrarchus labrax*) the exposure to high concentration of cadmium resulted in severe neuromast tissue damage and an altered escape behaviour in response to a water jet stimulus (Faucher et al., 2006). Also in a study with the banded kokopu (*Galaxias fasciatus*), cadmium exposed fish showed an alteration of rheotactic behaviour (ability to orientate in current), which was attributed to a disruption in the LL (Baker and Montgomery, 2001). In zebrafish, a link between loss of neuromast function and rheotaxis disruption has been established after copper treatment (Johnson et al., 2007); the time spent at equilibrium was reduced in zebrafish embryos exposed to copper.

These studies showed that besides observing toxic effects on the molecular and cellular levels, the LL system dysfunction has functional consequences in exposed organisms. It is therefore important to assess hair cell/neuromast dysfunction to detect subtle effects of chemicals.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Time of exposure</th>
<th>Age and Species</th>
<th>Description of disruption</th>
<th>Detection method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>5 µg/L</td>
<td>4h</td>
<td>adult sea bass</td>
<td>altered escape behaviour</td>
<td>escape behaviour in response to a water jet</td>
<td>Faucher et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>2 µg/L</td>
<td>72h</td>
<td>juvenile banded kokopu</td>
<td>alteration of rheotactic behaviour</td>
<td>rheotaxis observation</td>
<td>Baker and Montgomery (2001)</td>
</tr>
<tr>
<td>Copper</td>
<td>68, 244 µg/L</td>
<td>120h</td>
<td>120hpf zebrafish</td>
<td>disruption in rheotaxis (loss of neuromasts)</td>
<td>observation in water current</td>
<td>Johnson et al. (2007)</td>
</tr>
</tbody>
</table>

*Table 4.3: Effects of metals on mechanosensory behaviour*
4.4 In conclusion: linking developmental and toxicological studies

4.3.4 Further remarks

The range of chemicals that affect the LL organs might be broader than discovered so far. The toxicants that can affect the structure or function of the CNS and/or PNS, the so-called neurotoxicants (Tilson and Cabe, 1978; Tilson, 1993; Spencer et al., 1986), could as well affect the LL system of fish in general. In fact, exposure to neurotoxicants in humans can result in sensory, motor and cognitive dysfunction. Neurotoxicants like lead, methyl mercury, polychlorinated bisphenyls (PCBs) and environmental tobacco smoke affect the hearing system in humans (Lanphear et al., 2005). PCBs have also shown to accumulate in neuromasts of damselfish embryos living in PCB contaminated sites (Lobel and Davis, 2002). Recent investigations using crude oil exposure revealed effects on the sensory LL system in zebrafish as well (Kemadjou, J.R. in prep).

Very little is known on how aquatic toxicants impact the sensory system in aquatic organisms. The use of zebrafish neuromast as biological endpoint is not yet a standardized test in ecotoxicology. But as mentioned already, many exposure studies have shown the suitability of this system for assessing potential sensory- or neurotoxicants. In fact, chemicals are in direct contact with these superficial organs, which showed to be very sensitive. Dysfunction in sensory and/or nervous system can have dramatic impacts on survival of fish species since many different vitally important behaviours such as feeding, reproduction, predator avoidance and rheotaxis rely heavily on olfactory and sensory cues (Scott and Sloman, 2004; Blechinger et al., 2007; Whitlock, 2006). It is therefore crucial to study toxic effects on neuromasts in order to best evaluate the impact of chemicals in the aquatic environment. The findings gained with the zebrafish as model can be extrapolated to other fish species, as the basic architecture and function of the LL is highly conserved across teleosts (Webb, 1989).

4.4 In conclusion: linking developmental and toxicological studies

Having reviewed the abundant studies focusing on the neuromasts developmental process and the few investigations performed on neuromast disruption by toxicants, we propose the integration of both research fields. Potential links are explored below.

So far the toxicological analysis using neuromasts as endpoint assessed mainly the impact on hair cells differentiation. Two studies, intending to understand the process of hair cell regeneration, exploited the combination of chemical exposure with the molecular knowledge available. First study analysed the impact of metals (Hernandez et al., 2006b).
The expression of *atoh1a* and *eya1*, required for hair cell survival, was lost after copper treatment. The expression of *sox2*, a marker of supporting cells, was maintained. Indeed, the supporting cells were not affected by the treatment and could eventually differentiate into new hair cells after damage. Similarly, an up-regulation of *atoh1a* and *notch3* after neomycin treatment was detected, accompanied with an increase in supporting cells proliferation (Ma et al., 2008). The treatment, therefore, activated a cascade of effects that finally caused hair cell loss followed by hair cell regeneration. Hereby, the link between both research fields provided an excellent base for understanding chemicals impact on a cellular level.

Moreover, the unexploited possibilities to assess chemical impact in detail are tremendous, as not only the hair cell differentiation, but also the overall process of neuromasts development can be investigated. The effects of toxicants on the ability of hair cells to perform apical endocytosis could be assessed looking at the different genes essential for this process, such as the myosins, caveolins or cadherins. Similarly, the impacts of pollutants on the placode formation or general neurogenesis could be measured using the proneural genes, such as *ngn1* and *nrkd*. The damage caused to the primordium migration and cell deposition could be carefully assessed with *sdf1*, *cxcr4* and *cxcr7* gene expression.

The subtle effects of pharmaceuticals, industrial and agricultural chemicals and their by-products are only starting to be realised. Regulatory guidelines for aquatic pollutants are usually based on apical endpoints such as acute lethality, ignoring the effects appearing at much lower concentrations, so called "ecological death" as discussed in the review of Scott and Sloman (2004). The objective of their review was to point at the behaviour as an integrative tool, allowing to test the subacute effects of pollutants. Similarly, the process of neuromasts development enables to test the effects of chemicals on behaviour, on hair cell differentiation, and on the other specific developmental processes in the LL system, not investigated so far. This approach gives the opportunity to assess biological endpoints that occur at low, and therefore, more environmentally relevant, concentrations. Most importantly, the increasing range of molecular markers and genetic tools available in zebrafish enables to identify specific mode of action of potential toxic compound. So far, only a small part of the molecular and genetic potential of mechanosensory mutants and morphants has been exploited from toxicological research. There are multiple unexplored strategies available that integrate the basic knowledge on neuromast development with chemical exposure.

The potential of mechanosensory hair cell disruption to assess neurosensory impact of toxicants has only marginally been exploited to date. More effort should be invested in this direction. Deciphering sensory dysfunction caused by chemical exposure would
4.4 In conclusion: linking developmental and toxicological studies

require increasing the knowledge on the modes of action of these toxicants by further studying the normal mechanism of neuromast development. An integration of developmental and toxicological studies will provide a powerful tool for future risk assessment. The overall list of possibilities is far from being complete.
CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Estrogen receptors (ERs) research became mature the last fifty years mainly because of the key role of ERs in reproduction and breast cancer cells proliferation. In the last decade, a role of ERs also in non-reproductive functions in vertebrates was revealed. The findings of this thesis contributed to clarification of the role of ERs in non-reproductive processes, with a focus on the embryonic development and the development of the mechanosensory system.

ERs transcript expression during normogenesis

A first evidence of an importance of ERs for embryonic development was the presence of all three subtypes mRNA at already 4 hpf (chapter 2). This indication as well as the increased expression level of the three subtypes until 5 dpf, suggested the importance of ERs for early developmental processes. Even if all three subtypes were found to be present, the highest level of ERβ2 transcripts suggested a prominent role of this subtype in embryo development. In addition, the localization of ERβ2 mRNA revealed a high expression in the brain and in the neuromasts (chapter 3). The two other subtypes revealed to be locally less expressed, which confirms the above assumption. In order to identify brain regions that express ERβ2 transcripts, a preliminary experiment was performed using brain sections (Appendix A). Unfortunately, only weak background staining was detected due most probably to technical problems. More research is needed to identify embryonic
brain regions that express the different subtypes.

The presence of $\text{ER}_\beta_2$ in the brain and the neuromasts suggests already that this subtype may be the main mediator of estrogens action in these tissues and may have non-reproductive functions, such as being involved in embryonic development. Interestingly, in both mammals and fish, data on $\text{ER}_\beta$ rather than on $\text{ER}_\alpha$ are related to non-reproductive functions. In brain of adult rats, a clear distinction has been shown between the functional distribution of ERs. $\text{ER}_\alpha$ subtype is more expressed in the brain regions responsible for reproductive functions, while $\text{ER}_\beta$ is predominant in the extra-hypothalamic regions, which suggests its role beyond the hormonal-hypothalamic actions of the regulation of reproductive function.

Research on ERs expression pattern in juveniles brain, when sex differentiation takes place (around 20-40 dpf), would be of great interest. This would identify, which specific brain region expresses which ERs subtype and if there is a sex specific pattern, which would indicate further specific roles for each subtypes during this critical stage.

**ERs in the sensory system**

The knock down analysis of $\text{ER}_\beta_2$ confirmed the importance of this subtype in early development of zebrafish (chapter 3). The phenotype of the morphants corresponded to the typical phenotype of acoustic/vestibular mechanosensory mutants. After detailed analysis of $\text{ER}_\beta_2$ morphants, a role of this subtype in sensory hair cell differentiation has been demonstrated. Even if the roles of $\text{ER}_\alpha$ and $\text{ER}_\beta_1$ have not been investigated so far, the $\text{ER}_\beta_2$ morphants phenotype clearly indicates a mandatory presence of this latest subtype during neuromast development. These hair cells can be compared to the hair cells in the inner ear of mammals. In fact, the ERs expression has also been found in the hair cells of mice ear and $\text{ER}_\beta\text{ko}$ mice were deaf. This suggests that ERs play a conserved role among vertebrates in this sensory system. In order to further support the role of $\text{ER}_\beta_2$ in hair cell differentiation, an experiment was performed trying to copy the knock down phenotype by blocking the receptors chemically with an unspecific ER antagonist, ICI 182,780. Unfortunately, no phenocopy was observed as described in Appendix B.

Beside the mechanosensory/auditory system, ERs have been detected in the preoptic area of adults zebrafish brain (Menuet et al., 2002). Interestingly, this study also revealed a weak expression of $\text{er}_\beta_1$ and $\text{er}_\beta_2$ in the eye. Further localization analysis is required to confirm this expression. Studies in mammals, however, support these findings by showing ERs expression in the eye and, moreover, in the olfactory bulbs (Shughrue et al., 1997; Kato et al., 2006). In humans, $\text{ER}_\alpha$ protein was detected in the retina and retinal pigment
General conclusions and future perspectives

The epithelium of young women eyes (Ogueta et al., 1999). Altogether, the ERs role in the sensory system could be broader as discovered so far and research on ERs function in the olfactory system and vision would be an interesting field for future investigations.

Neuromasts as a biological marker

ER,β2 localization and function in neuromasts attracted our interest to these organs. Nowadays, neuromasts are intensively used to study the mechanisms that cause hearing loss. This field of research has developed because of the conservation of morphological, electrophysiological and biochemical properties between neuromasts and inner ear of higher vertebrates.

However, in ecotoxicology, the neuromasts are not systematically used to detect disruption in the mechanosensory system of fish till now. Our literature review (chapter 4), combining the research performed on the mechanisms of lateral line development with the studies looking at chemical impacts on neuromasts, demonstrated the great potential of these organs for testing toxicity on an non-apical biological endpoint.

In environmental risk assessment, very few tools are available for testing the effects of chemicals on the sensory system, mainly because of the difficulty to assess biological endpoints, such as behaviour, as well as the time needed before behavioural effects can be assessed. Many vital behaviours depend on the sensory capacities of the fishes. In some teleost, prey capture, predator avoidance, orientation in water, but also the orientation within swarms mainly depends on functional lateral line organs. If environmental pollutants, including pharmaceuticals, heavy metals or xenoestrogens, disrupt this system, the overall behaviour of the individual species with consequences for growth, development and reproduction, might be impacted. It is, therefore, important to develop tools for assessing chemical effects on this sensory system, as it is crucial for survival and biodiversity.

Moreover, the molecular tools available in neuromasts, such as gene markers and reverse genetics, may allow to study specific mode of action of chemical compound affecting the mechanosensory system. This knowledge is indeed very important, as many biological impacts could be predicted on the basis of these findings. Therefore, a need to further investigate the impacts of chemicals in non-apical endpoints, such as the mechanosensory system, is crucial.
ERs in other signalling pathways

Estrogen signalling is one of the most studied signalling pathways. Recent research showed to be more complex than just binding of the hormone to the corresponding receptor (genomic pathway). An additional non-genomic pathway was identified, where membrane receptors and intracellular second messengers are involved. ERs are also able to indirectly associate with promoters through protein-protein interactions with other DNA-binding transcription factors, the so called ligand independent actions of ERs (Klinge et al., 2004; O’Lone et al., 2004). Consequently, estrogen signalling can modulate the activity of various signalling pathways in target cells by different mechanisms ranging from genomic, non-genomic, as well as ligand dependent and independent pathways.

In this thesis we added to the complexity of estrogen signalling by showing that a cross-talk with yet another signalling pathway is possible. The six major universal signal transduction, responsible for the basic machinery for cell fate transition in embryonic development are the Hedgehog, bone morphogenetic proteins, Wingless/int1 (Wnt), steroid hormone receptor, Notch and receptor tyrosine kinase (Martinez Arias and Stewart, 2002). A cross-talk between the Wnt and Notch pathway has been identified (Hayward et al., 2008). The androgen receptor has also been showed to interact with the Notch signalling pathway (Belandia et al., 2005). In this thesis, it was shown for the first time, that estrogen signalling is able to interfere with the Notch signalling pathway, participating in this way in cell fate decision (chapter 3). The knock down of ERβ2 activated the expression of two members of the Notch family, and consequently disturbed the hair cell differentiation of neuromasts. Further investigation has, however, to be conducted in order to understand this cross-talk.

One potential explanation on why estrogen signalling has such a pleiotropic impact on cells, might be found in studies on the evolution of vertebrate steroids receptors (Thornton, 2001). As estradiol is the last to be synthesised in the metabolic pathway of steroids, the receptor might have evolved before the hormone itself, as an independent transcription factor (Maggi et al., 2004). Thus, the unliganded ER could be activated by cascades of different signalling molecules triggered by membrane receptors, and in this way control cell metabolism. When, during evolution, estradiol was synthesised and began to control ER activity, it is possible that the receptor has still maintained some of its original functions, which would explain why estradiol, and not other steroids, may exert such a variety of diversified effects in so many different target tissues.

In the particular case of estrogens and the Notch signalling, a very recent study using breast cancer cell lines, revealed a similar up-regulation of the Notch signalling pathways
after blocking the ERs with tamoxifen (Rizzo et al., 2008). A combined treatment using Notch inhibitors and ER antagonist was proposed to avoid the side effects of tamoxifen and therefore, avoid the proliferation of breast cancer cells. Using the zebrafish lateral line system to study in more details the interaction between the Notch receptor and the ERs could help to decipher the mechanisms behind and deliver new therapeutic strategies to ameliorate the treatment of cancer patients. Moreover, this cross-talk could have a broader impact in endocrine disruption. Xenoestrogens could alter the overall cell fate decision of organ development and cause long-term effects in organisms. Although no direct evidence has been found so far, an interesting field of investigation is open for future investigators.

**ERs in endocrine disruption**

The xenoestrogens impact has been shown on ER transcripts and revealed to regulate one of the three subtypes very early in development (chapter 2). ER\(\alpha\) may be the subtype that mediates the effects of endocrine disruptors. One of these effects is the well-known up-regulation of the biomarker gene *vitellogenin*. Endocrine disruptors are able, in this way, to affect early development and cause major problems in wildlife development and long-term impacts on the population. A mechanistic approach is needed to confirm that the major mediator of endocrine disruption in early life stage is ER\(\alpha\).

The regulation of each subtype has been investigated on extracts from whole embryos. Additional experiments would be important for discovering tissue specific regulation of ERs in zebrafish embryos. These results would also clarify whether the neuromasts could be affected by endocrine disruptors, as with the data performed on whole extracts, the dominant subtype in neuromasts showed to be slightly or not affected by EE\(_2\) exposure and the strong regulated subtype is very weakly detectable in these organs. Consequently, it is possible that xenoestrogens do not affect these organs, but if xenoestrogens inhibit locally ER\(\beta_2\) expression in the neuromasts, the mechanosensory system of embryos could be disrupted. In this case, neuromasts could be a novel target of endocrine disruption. Nevertheless, EE\(_2\) exposure did not cause embryos to circle, which suggests that the hair cells of treated embryos were not affected.

Furthermore, investigation is needed to detect ER subtypes at critical developmental time points, such as the time where sexual differentiation takes place, between 20 and 40 dpf. Very little is known on the impact of xenoestrogens on the PNS in juveniles and adults and EDCs impact on the sensory system is mainly investigated in birds.
Concluding remarks

This thesis focused on the importance of ERs in early vertebrate development. Its major finding was ERs’ detection at the embryogenesis of zebrafish and their determinant role during the development of the mechanosensory system organs. Knock-down experiments on the neuromasts, provided insights about the potential use of these organs (part of the lateral line system) as toxicological endocrine disruptive endpoints. More generally, results also suggested that neuromasts may allow assessing the impact of other chemicals in fish. Finally, because the studied cellular processes are conserved among vertebrates (fish and mammals), ERs findings gave not only insights in aquatic ecotoxicology, but suggesting new approaches to study cancer or hearing diseases in humans.


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A.1 Introduction

Estrogens are known to be essential for neuronal growth and differentiation in the central nervous system (CNS). ERβ knock out (ko) mice and ERβ localization studies allowed insights into the specific role of this subtype in the CNS. A function of ERβ in neuronal survival and development has been revealed. Interestingly, the ERβ ko mice embryos had fewer neurones in the cortex and showed a degeneration of neuronal cell bodies throughout the brain (Wang et al., 2001). Also in human embryonic brain cells, an expression of ERβ was detected (Fried et al., 2004). Lower vertebrates like zebra finch embryos express ERs in the diencephalic regions of the developing brain (Perlman and Arnold, 2003).

In this work, zebrafish was used as model organism in order to find out which brain regions expressed ERs in early development. So far ERs distribution has only been described in adult zebrafish brain. An expression of ERs mRNA has been detected in preoptic regions, mediobasal hypothalamus and posterior tuberculum (Menuet et al., 2002). As described in chapter 3, ERβ2 transcripts were found to be strongly expressed in developing brain using in situ hybridization (ISH) in whole mount embryos. In order to identify more specifically the brain regions where ERβ2 mRNA is expressed, two kinds of ISH methods were used, the fluorescent ISH (FISH) on whole mount embryos and ISH on brain sections.
A.2 Methods

A.2.1 whole mount FISH

The same $er\beta_2$ antisense probe was used as described in chapter 3 and EAAT, glutamate transporter, provided by Stephan Neuhauss laboratory, was used as positive control. Larvae of 72-96 hpf were dechorionated and fixated in 4% Paraformaldehyde (PFA) in PBS. Then they were dehydrated in MetOH and rehydrated through graded MetOH to PBS, incubated for 40-45 min at 37°C in Proteinase K (5 µg/ml in PBS), briefly rinsed in PBT and then refixed in PFA. The embryos were, then, prehybridized for 4 h at 55°C and then hybridized overnight in HYBE (50% formamide, 5xSSC, heparin 50 mg/l, 0.1% Tween20, tRNA 10 mg/l, citric acid 1M). After several washes with high stringency, the embryos were washed with blocking buffer and then incubated with preabsorbed anti-digoxigenin(DIG)-rhodamine overnight in the dark at 4°C. Finally, the embryos were observed under fluorescent microscope.

A.2.2 ISH on paraffin sections

The same $er\beta_2$ antisense probe was used as described in chapter 3 and the $ngn1$ antisense probe was used as positive control (see chapter 3). The protocol used was similar to the one described previously (Goto-Kazeto et al., 2004). Paraffin sections (5 µm thickness) were deparaffinised with xylene, rehydrated through graded ethanol (100%-70%) to DEPC water, incubated 10 min at 37°C in proteinase K (5 µg/ml in PBS), briefly rinsed with DEPC water, acetylated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min, and dehydrated through graded dilutions of ethanol. The sections were then prehybridized for 3 h in HYBE at 54°C. For hybridization, the labelled riboprobes were added to the pre-warmed HYBE (4 ng/µl). After the hybridization overnight at 54°C in a humidified chamber, the slides were washed with high stringency, dehydrated, dried and observed under bright field microscope.

A.3 Results and Discussion

The fluorescent probe showed no specific staining in whole mount embryos (Figure A.1). A weak dot-shaped staining was detected in the middle of the brain section, which is most probably weak background staining (Figure A.2). None of these two methods delivered successful results when applied to 72 hpf embryos brain using the same probe as in chapter 3, where $er\beta_2$ expression was detected in the neuromasts and the head. The positive
controls did not deliver any detection in both procedures as well. For this reasons the problem was most probably due to the anti-DIG antibody conjugated to rhodamine, maybe a problem from the manufacture, as this protocol was nearly identical to the one used in chapter 3. However, the negative results on brain sections might come from a problem with tissue preparation or with the technique used. Thus, further experiments would be needed to identify the brain regions that express ERs in embryos.

Figure A.1: Localization of erβ2 mRNA with fluorescent in situ hybridization in 72 hpf larvae. Specific antisense probe hybridization was not detected.

In zebrafish, also the mRNA expression of the aromatase producer enzyme, cyp19b, has been detected in the E2 exposed developing brain, suggesting estrogens role in early development of this organ (Menuet et al., 2005). In addition, E2 induction of the cyp19b gene in zebrafish embryos was blocked by an excess of the pure ER antagonist ICI 182, 780, as shown with RT-PCR (Kishida and Callard, 2001), indicating that functional ERs were involved in this process.

A broader knowledge of ERs expression is needed to decipher ERs role in brain development as developmental perturbations of estrogen signalling in the brain may cause permanent changes in neural architecture in adults.
Figure A.2: Localization of $er\beta_2$ mRNA with in situ hybridization on sections in 72 hpf larvae. Antisense probe was not detected in brain sections, while weak background staining is indicated by black arrows.
APPENDIX B

EFFECTS OF ER ANTAGONIST
ICI 182, 780 ON ZEBRAFISH EMBRYOS

B.1 Introduction

Blocking the ERβ2 impaired zebrafish embryos development (see chapter 3). The hair cells of the morphants were not differentiated, which caused embryos to circle. This observable phenotype could further be used to screen for chemicals that block ERβ2. For this reason we performed a phenocopy experiment by blocking ERs with a complete estrogen antagonist. ICI 182, 780 (ICI) is a steroidal pharmaceutical agent designed to replace tamoxifen in breast cancer therapy (Howell et al., 2000). Indeed, ICI is a pure anti-estrogen, whereas tamoxifen, a non-steroidal compound, showed to act only as partial antagonist in certain tissues. Studies examining the mode of action of ICI have demonstrated that the ability of the ER to activate or inhibit transcription in a ligand dependent or independent manner in vivo is completely blocked by ICI (Parker, 1993; Dauvois et al., 1993). Multiple changes in ER function after ICI treatment appear to contribute to the blockade of estrogen action. These include impaired dimerization, increased turnover, and disrupted nuclear localization. Thus, not only is ER blocked functionally, but cellular levels of ER are reduced markedly by ICI. Also the transcription of ER-regulated genes is inhibited as a consequence of the "down-regulation" of ER by ICI 182 780, thus avoiding any estrogenic side effects (Genissel and Carreau, 2001). In vitro studies revealed that ICI is able to block both ER subtypes, ERα and ERβ (Sun et al., 2002). Even if this compound might be able to block the two other zebrafish ER
subtypes, we hypothesized that embryos exposed to ICI will show a phenotype similar to ERβ2 morphants, as ERβ2 is the most expressed subtype during early developmental stages (see chapter 2).

B.2 Methods

B.2.1 Sample collection

The eggs were collected one hour after the daylight. After pooling from the different aquaria, they were washed with an E₃ medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄). The eggs were raised until 72 hpf in E₃ with or without ICI and phenotype was compared under the stereomicroscope between exposed and control samples.

B.2.2 ICI exposure

The stock solution was made with 10⁻² M ICI 182, 780 dissolved in 100% EtOH. The solvent control solution was made with 100 ml E₃ and EtOH to a final concentration in the solution of 0.01%. To obtain the final ICI dilution of 10, 100, 1000 ng/L, a working dilution of 100µg/L was made in order to obtain a concentration of solvent which does not exceed 0.01% EtOH in a volume of 100 ml E₃. All glassware was rinsed with acetone and incubated overnight in exposure solution in order to saturate the glass surface. After fertilization (1 hpf) the eggs were distributed in glass Petri dishes at a density of 60 per 40 ml of solution. After 24 hpf the unfertilized eggs were removed and every 24 hpf the medium was renewed in order to maintain the ICI concentration.

B.3 Results and Discussion

There were no biological defects observed in embryos treated with all ICI concentrations compared to solvent control and blank (E₃). Different reasons could be at the origin of these negative results. Some studies revealed that ICI might selectively block ERα and not ERβ, as shown in rat male reproductive tract (Oliveira et al., 2003) and in mammalian cell lines (Larsen et al., 1997; Singh et al., 2000). In the Atlantic croaker, ERα and ERβ₁ have a greater affinity towards ICI than towards estradiol, whereas ERβ₂ showed higher relative binding affinities for estradiol than for anti-estrogens such as ICI (Hawkins and Thomas, 2004). These differences are most probably due to distinct conformational changes induced by ICI in each ERs subtype (Paige et al., 1999).
The concentrations used might not be sufficient for inducing adverse effects in zebrafish embryos development. This is unlikely, as in the sea urchin, a developmental alteration has been reported when exposing embryos to 30 ng/L of ICI (Roepke et al., 2005).

Altogether, it is possible that the missing ERβ2 morphants phenotype in embryos exposed to ICI, is due to a relative insensitivity of ERβ2 to this estrogen antagonists. The high affinity of ICI towards ERα might not be sufficient to cause adverse biological effects, as this subtype protein might occur in low levels (low mRNA levels measured) in early development. However, it would be interesting to know whether molecular changes in regard to ERα can be measured in treated larvae. More research is, therefore, needed to decipher the impact of ICI on zebrafish embryos.