

From the DEPARTMENT OF BIOSCIENCES AND NUTRITION

Karolinska Institutet, Stockholm, Sweden

**ZEBRAFISH AS A MODEL TO STUDY THE  
NEUROENDOCRINE SYSTEM AND TOXICITY OF  
ENDOCRINE DISRUPTORS**

Gayathri Chandrasekar



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*To my beloved mother*

*karmany evadhikaras te  
ma phalesu kadacana  
ma karma-phala-hetur bhur  
ma te sango 'stv akarmani*

*- Bhagavad Gita*

Translation

You have a right to perform your duty, but you are not entitled to the fruits of action  
Never consider yourself to be the cause of the results of your activities  
And never be attached to not doing your duty

First they ignore you,  
Then they laugh at you,  
Then they fight you,  
Then you win

*-Mahatma Gandhi*



## ABSTRACT

Zebrafish is a popular vertebrate model system to study development and perform genetic analysis. It offers numerous advantages such as small size, short generation time, high fecundity, rapid and *ex utero* development of embryos and optically transparent embryos. Zebrafish is genetically closely related to humans and share high similarity in developmental processes, physiology and behavior. In addition, recent advances in forward and reverse genetics coupled with the availability of a large number of mutants makes zebrafish a useful model for genetic analysis of development. Furthermore, the above mentioned advantages offered by this model organism will be a valuable resource for toxicologists not only to assess toxicity of endocrine disrupting chemicals (EDCs) but also to dissect the mechanisms of toxicity of EDCs.

The development and function of the neuroendocrine system in zebrafish is to a great extent similar to other vertebrates. Thus, in an aim to understand the development of the neuroendocrine stress axis we have characterized the expression of *corticotropin-releasing hormone (crh)* in the embryonic brain of zebrafish. Transcripts of *crh* were detected in a broad range of adult tissues and also during embryonic and larval stages. Whole-mount in situ hybridization analysis revealed distribution of *crh* transcripts in various regions of the developing brain including telencephalon, preoptic area, hypothalamus, posterior tuberculum, thalamus, epiphysis, midbrain tegmentum, hindbrain and retina. Expression of *crh* in the preoptic area and in extra-hypothalamic regions is consistent with its roles as a hypophysiotropin and a neuromodulator.

Estrogen receptors are ligand activated transcription factors involved in regulating the neuroendocrine axis of reproduction. We have analyzed the mRNA levels of the *esr* genes in the absence and presence of exogenous ligands. The three *esr* genes, *esr1* (ERalpha), *esr2b* (ERbeta1) and *esr2a* (ERbeta2) were expressed in all adult tissues tested in the absence of exogenous ligands. Expressions of *esr1* and *esr2a* were altered in the liver, brain, testis and intestine following ligand treatment (17 $\beta$ -estradiol (E2) or diethylstilbestrol (DES) or 4-nonylphenol, (4-NP)). During embryogenesis, only *esr1* and *esr2b* were predominantly expressed and both were regulated by ligands. Our results demonstrate that the *esr* genes in zebrafish are regulated in a sex- and tissue specific manner and that 4-NP, a well-known endocrine disruptor possess both agonist and antagonist properties in adult tissues.

We further extended our study to assess the toxic effects of 4-NP on developing embryos and larvae of zebrafish. A sub-lethal dose of 4-NP not only perturbed the neuroendocrine axis but also induced distortions/kinks and herniations in the notochord. The differentiation of the notochordal cells and the formation of the perinotochordal basement membrane were disrupted by 4-NP. Early disturbances induced by 4-NP in the notochord resulted in deformities in the vertebral column at late larval stages. The notochord phenotype was accompanied by impaired swimming pattern. Repeated electrical stimulation of the larval muscles of 4-NP treated embryos showed impairment in the relaxation between stimuli which might be a possible reason for the defective swimming observed in 4-NP treated embryos.

## LIST OF PUBLICATIONS

- I. **Gayathri Chandrasekar**, Gilbert Lauter, Giselbert Hauptmann. Distribution of corticotropin-releasing hormone in the developing zebrafish brain. **J. Comp. Neurol.** 2007; 505(4): 337-351
  
- II. **Gayathri Chandrasekar**, Amena Archer, Jan-Åke Gustafsson, Monika Andersson Lendahl. Levels of  $17\beta$ -estradiol receptors expressed in embryonic and adult zebrafish following *in vivo* treatment of natural or synthetic ligands. **PloS One** 2010; March 12; 5(3): e9678
  
- III. **Gayathri Chandrasekar**, Anders Arner, Satish Srinivas Kitambi, Karin Dahlman Wright, Monika Andersson Lendahl. The estrogenic compound 4-nonylphenol disrupts the notochord morphogenesis in zebrafish.  
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# TABLE OF CONTENTS

1	Introduction	
	1.1 The neuroendocrine system	1
	1.2 The hypothalamic-pituitary-adrenal/interrenal axis	2
	1.3 The hypothalamic-pituitary-gonadal axis	4
	1.4 Estrogen receptors	5
	1.5 Estrogen actions in the neuroendocrine system and other areas of the brain	8
	1.6 Endocrine disruptors	11
	1.6.1 Diethylstilbestrol	12
	1.6.2 4-nonylphenol	12
	1.7 Effects of EDCs on the neuroendocrine system	14
	1.7.1 The HPG axis	15
	1.7.2 The thyroid hormone axis	16
	1.7.3 The HPA axis	16
	1.7.4 The growth hormone axis	17
	1.8 Estrogen receptors in fish	17
	1.9 Zebrafish as a model organism	18
	1.9.1 The zebrafish HPI axis development	19
	1.9.2 Estrogen receptor subtypes in zebrafish	21
	1.9.3 Assessment of toxicity and endocrine disruption using zebrafish	22
2	Aims	24
3	Materials and Methods	25
4	Results and Discussion	28
5	Conclusions	35
6	Acknowledgements	37
7	References	39

## LIST OF ABBREVIATIONS

4-NP	4-nonylphenol
ACTH	Adrenocorticotropin hormone
AR	Androgen receptor
AP-1	Activator protein 1
CRH	Corticotropin releasing hormone
CRF	Corticotropin releasing factor
ChAt	Choline acetyl transferase
CYP19a2	Aromatase brain isoform
DBD	DNA binding domain
DES	Diethylstilbestrol
DMSO	Dimethylsulfoxide
DA	Dopaminergic neurons
dpf	Days post fertilization
E1	Estrone
E2	17 $\beta$ -estradiol
E3	Estriol
EE2	17 $\alpha$ -ethinylestradiol
ER	Estrogen receptor
ERE	Estrogen response elements
ER $\alpha$ KO	Estrogen receptor $\alpha$ knock-out mouse
ER $\beta$ KO	Estrogen receptor $\beta$ knock-out mouse
EDC	Endocrine disrupting chemicals
FSH	Follicle stimulating hormone
GR	Glucocorticoid releasing hormone
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GnRH	Gonadotropin releasing hormone
GTH $\alpha$	Gonadotropin $\alpha$
hpf	Hours post fertilization
HPA	Hypothalamic-pituitary-adrenal axis
HPI	Hypothalamic-pituitary-interrenal axis
HPG	Hypothalamic-pituitary-gonadal axis

LBD	Ligand binding domain
LH	Luteinizing hormone
MSH	Melanocyte stimulating hormone
NLT	Nucleus lateralis tuberis
NPE	Nonylphenol ethoxylates
NPO	Nucleus preopticus
PCB	Polychlorinated biphenyl
POMC	Pro-opiomelanocortin
PR	Progesterin receptor
PRL	Prolactin
PVN	Paraventricular nucleus
SF-1	Steroidogenic factor 1
Sp1	Specificity protein 1
SS	Somatostatin
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TR	Thyroid hormone receptor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
uts 1	Urotensin 1
uts 3	Urotensin 3
VTG	Vitellogenin
ZRP	Zona radiata protein



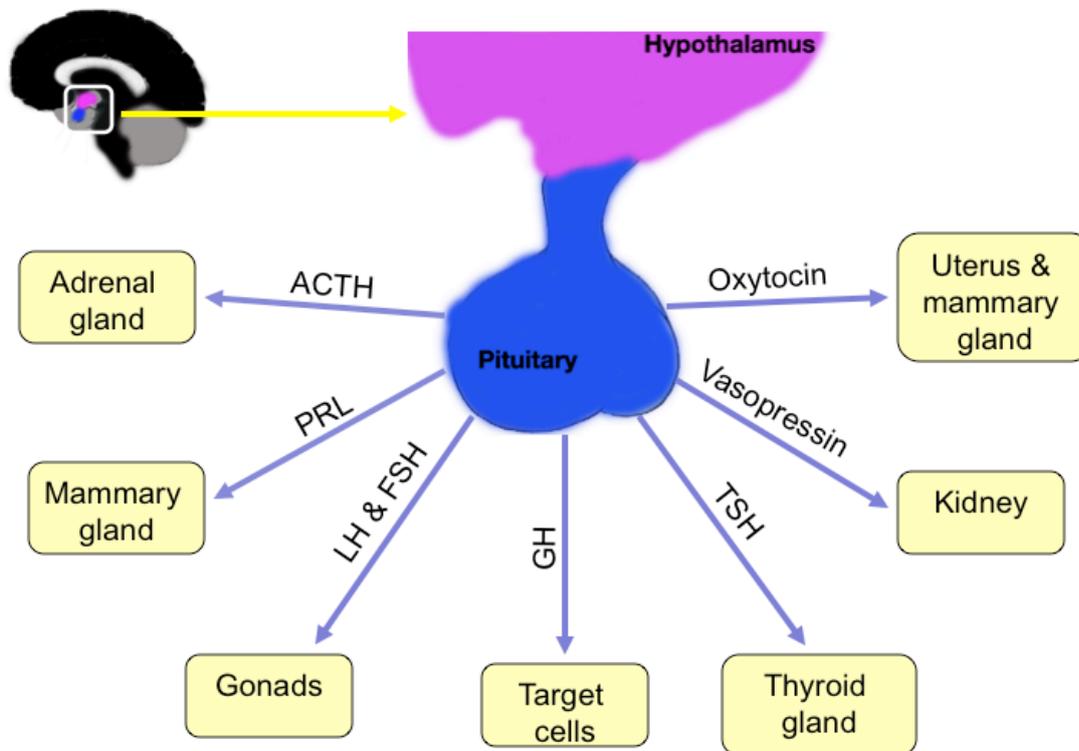
# 1. INTRODUCTION

## 1.1 The neuroendocrine system

The neuroendocrine system includes the central nervous system and the endocrine glands which interact to regulate the physiological functions in living organisms. The pituitary gland, which is located at the base of the hypothalamus, constitutes an interface connecting the central nervous system and the endocrine glands. Secretion of the pituitary hormones are regulated by neurohormones produced in the hypothalamus. The pituitary gland is composed of three lobes which are termed pars distalis (anterior lobe), pars intermedia (intermediate lobe) and pars nervosa (posterior lobe). Together the anterior and intermediate lobes are referred to as the adenohypophysis. The adenohypophysis secretes growth hormone (GH), adrenocorticotropin hormone (ACTH), Thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and melanocyte stimulating hormone (MSH). Pituitary hormones TSH, ACTH, LH, FSH stimulate the release of endocrine hormones from the thyroid gland, adrenal gland and ovary respectively. MSH, PRL and GH act on non-endocrine targets in the brain and other parts of the body. Pars distalis stores the hypothalamic hormones, oxytocin and vasopressin.

Hypothalamus, in addition to synthesizing hormones, performs other functions such as controlling body temperature, circadian rhythm, electrolyte balance, hunger, thirst and emotional behaviour. The hypothalamic hormones are produced in the neurosecretory cells of the hypothalamus. There are two types of neurosecretory cells: the larger magnocellular cells and the smaller parvocellular cells. Oxytocin and vasopressin are synthesized in the magnocellular cells and are released into the neurohypophysis of the pituitary through the axon terminals of the magnocellular neurons. The parvocellular cells that synthesizes corticotropin releasing hormone (CRH), thyrotropin releasing hormone (TRH), somatostatin (SS), gonadotropin releasing hormone (GnRH) and growth hormone releasing hormone (GHRH) project their axons to the median eminence where they release the hormones into the hypophyseal portal veins. A small number of parvocellular cells also produce vasopressin which in combination with CRH act on the anterior pituitary to secrete ACTH. The adenohypophyseal hormones, ACTH, TSH, LH and FSH are regulated by hypothalamic releasing hormone whereas the other three adenohypophyseal hormones,

PRL, GH and MSH are regulated individually by one stimulatory and one inhibitory hypothalamic hormone (Fig 1).

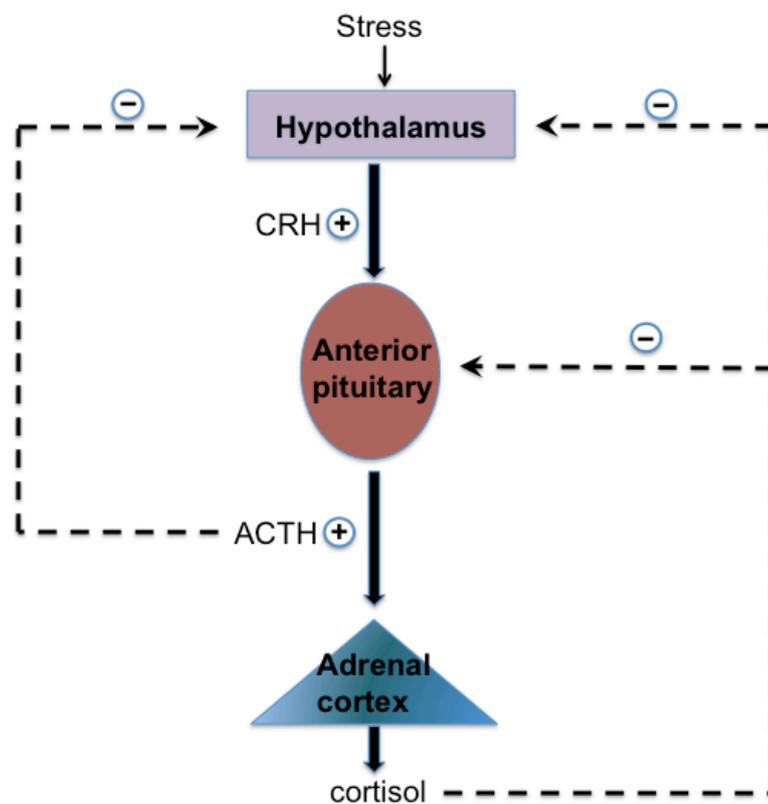


*Fig 1: Schematic representation of the neuroendocrine system. The Pituitary gland acts as an interface connecting the hypothalamus to the endocrine glands. Oxytocin and vasopressin are the two hypothalamic hormones that are stored in the posterior lobe of the pituitary. ACTH, TSH, LH, FSH, GH and PRL are the hormones produced in the anterior lobe of the pituitary gland. MSH and  $\beta$ -endorphin are produced in the intermediate lobe of the pituitary (not shown).*

## 1.2 The hypothalamic-pituitary-adrenal/interrenal axis

The hypothalamic-pituitary-adrenal axis (HPA) controls the physiological reactions to stress. In response to stress, CRH is released from the paraventricular nucleus (PVN) of the hypothalamus into the median eminence. It is then transported to the anterior pituitary where it stimulates the corticotropes to synthesize proopiomelanocortin (POMC) which is further cleaved to produce ACTH. CRH can synergize with vasopressin to stimulate the release of ACTH. ACTH then acts on the adrenal gland to stimulate steroidogenesis. The steroid hormone cortisol (corticosterone

in rodents) is the primary stress hormone in humans which regulate biochemical and physiological changes to cope with stress. Feedback systems are an important aspect of the hormones of the hypothalamus, the pituitary and the endocrine glands. Cortisol can negatively feedback to the pituitary and the hypothalamus to inhibit the production of ACTH and CRH respectively. A short feedback loop is also provided by ACTH to the hypothalamus and other areas of the brain to inhibit CRH secretion (Fig 2). In the brain, CRH is also present in extra-hypothalamic regions where it acts as a neuromodulator regulating autocrine, electrophysiological and behavioral functions (De Souza and Grigoriadis 1995; Dunn and Berridge 1990).



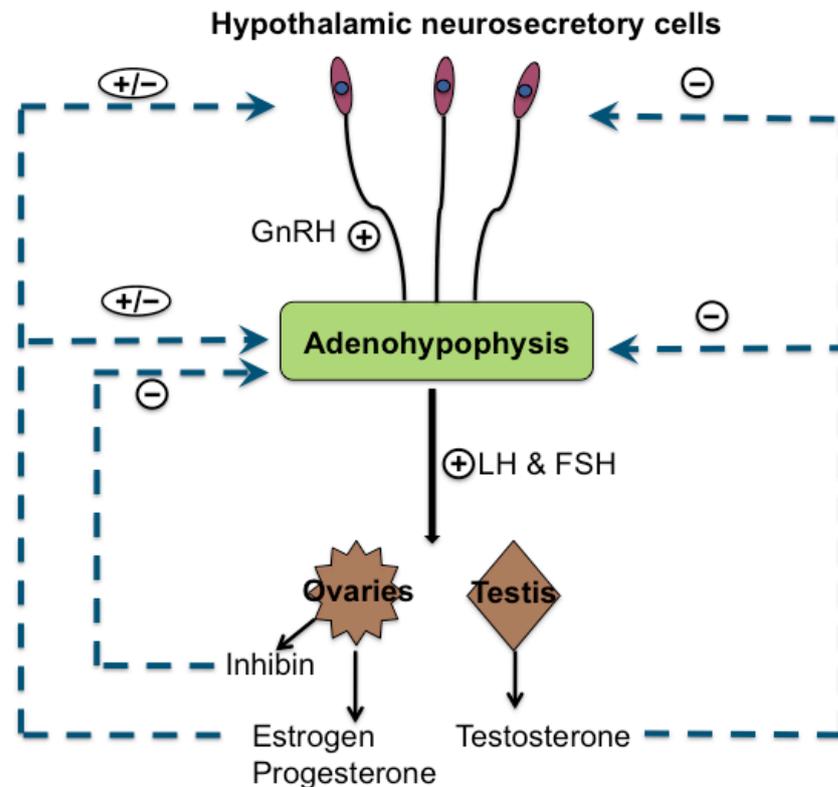
**Fig 2: The hypothalamic-pituitary-adrenal axis with its feedback loops.** The hypothalamic releasing hormone CRH stimulates the corticotrophs located in the adenohypophysis to produce ACTH which acts on the adrenal gland to secrete cortisol. Cortisol regulates the body to cope with stress. The HPA axis is strictly controlled by feed back loops acting at the level of the hypothalamus and the pituitary gland.

The adrenal gland in teleosts is known as the interrenal gland and the stress axis is called the hypothalamic-pituitary-interrenal axis (HPI). The functioning of the stress axis in fish is very similar to humans. The interrenal gland is located in the

head kidney and cortisol is the major corticosteroid secreted by the interrenal cells (Chester-Jones and Morsley 1980). In teleosts, the nucleus preopticus (NPO), homologous to the mammalian PVN contains the neurosecretory cells that synthesize CRH. CRH immunoreactivity has also been observed in the nucleus lateralis tuberis (NLT) in some fish species. The neurons of these hypothalamic nuclei directly project their axons to the pituitary gland as teleosts lack the median eminence (Fellmann et al., 1984; Jorgensen and Larson 1967). Similar to mammals, the HPI axis can also be activated by arginine vasotocin which is orthologous to the mammalian vasopressin.

### **1.3 The hypothalamic-pituitary-gonadal axis**

The hypothalamic-pituitary-gonadal axis (HPG) controls development and reproduction. The release of LH and FSH from the gonadotropes are stimulated by hypothalamic GnRH. In females, both LH and FSH trigger the ovaries to produce estrogen, progesterone and inhibin. In males, LH acts on the testes to produce testosterone and FSH acts on the sertoli cells to produce inhibin. The HPG axis with the feedback system is illustrated in Fig (3). Estrogen, one of the major components of the HPG axis is found at higher levels in females than in males. Estrogen promotes secondary sexual characteristics in females whilst in males it is required for the maturation of sperm. The biological functions of estrogens are mediated through estrogen receptors.

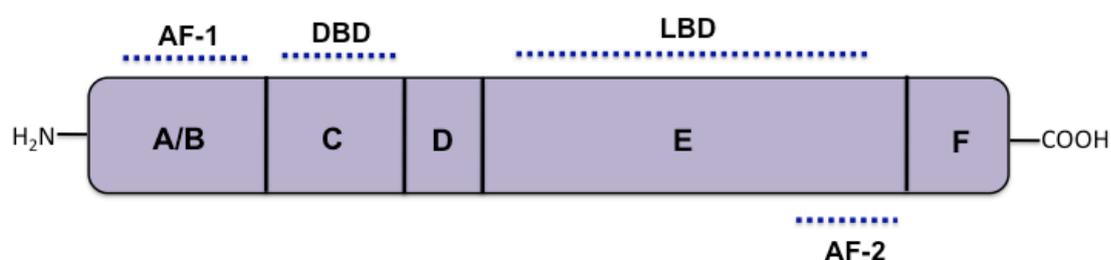


**Fig 3: The hypothalamic-pituitary-gonadal axis with its feedback loops.** GnRH released from the hypothalamus stimulates the pituitary gonadotrophs to produce LH and FSH. In females, LH and FSH stimulate the ovaries to release estrogen, progesterone and inhibin. In males, LH activates the testis to produce testosterone which exerts a negative feedback on the hypothalamus and the pituitary. Estrogen and inhibin have a negative feedback on FSH. Estrogen also has a positive feedback on LH release. Progesterone has a negative feedback on both LH and FSH.

#### 1.4 Estrogen Receptors

Estrogen receptors (ERs) are members of the nuclear receptor family of transcription factors. Like other nuclear receptors, ERs have six distinct domains (Fig 4). The A and B domains at the N-terminal region are not conserved and consists of the ligand independent transactivation domain AF-1. The highly conserved DNA-binding domain (DBD; the C domain) is involved in binding to specific sequences on DNA known as estrogen response elements (ERE). The DBD is linked to the ligand-binding domain (LBD; the E domain) by a poorly conserved D domain. The LBD at the C-terminal consists of a ligand binding pocket, a dimerization interface, recognition sites

for co-activators and co-repressors and the ligand-dependent transactivation function 2 (AF-2) (Nilsson et al., 2001).

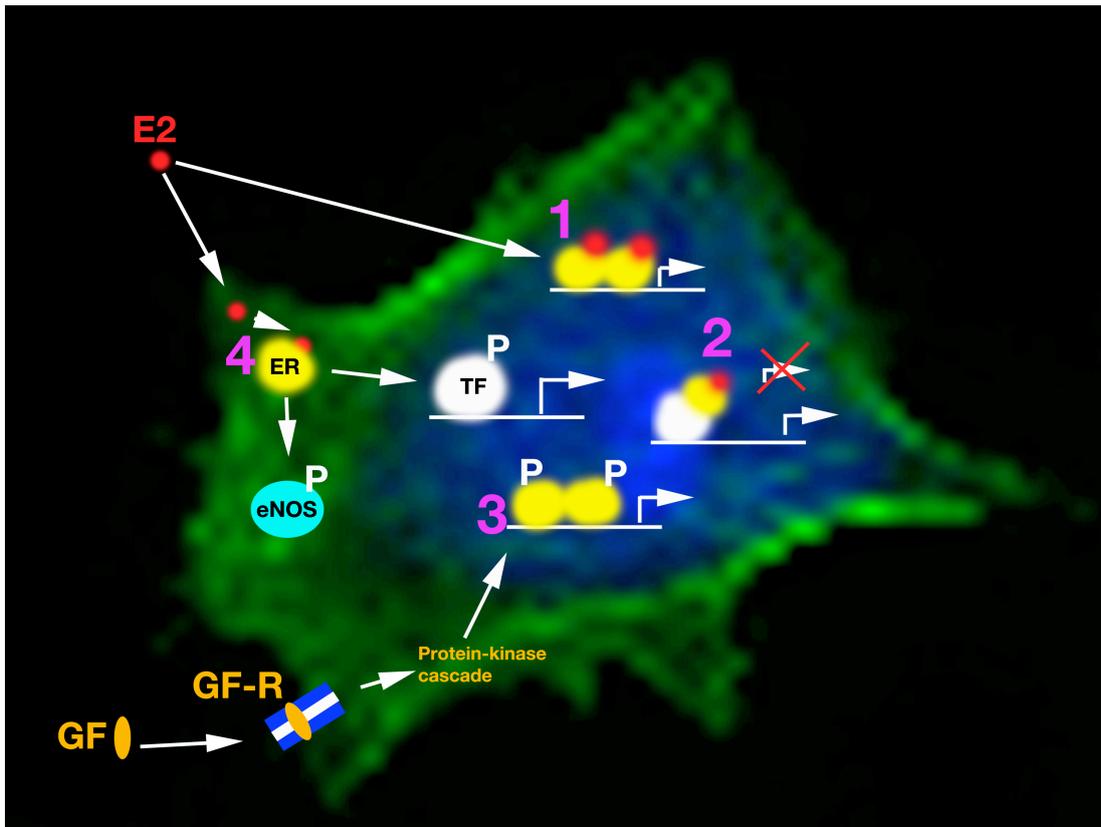


**Fig 4: Diagrammatic illustration of the domains of the estrogen receptor.** The A/B domain contains the ligand-independent AF-1 transactivation site. C and E domains are the DNA binding and the ligand binding domains respectively. The LBD contains the ligand-dependent AF-2 transactivation site.

There are two forms of ERs in mammals, ER $\alpha$  and ER $\beta$  encoded by distinct genes. ERs share a high degree of sequence similarity, particularly in their DBDs and LBDs and can bind to 17 $\beta$ -estradiol (E2) with similar affinities (Nilsson et al., 2001). However, numerous studies have reported that the ER subtypes have different affinities for some natural and synthetic ligands (Sun et al., 1999; Kuiper et al., 1998a). Variation in the amino acid residues between the two ERs at the N-terminal end may be one possible explanation for their diversity in binding and responsiveness to different ligands. Analysis of mRNA levels of the ER subtypes in different species show variability in their tissue distribution suggesting that they might have diverse roles in the body (Hawkins et al., 2005; Kuiper et al., 1998b; Meneut et al., 2002). Additionally, knockout studies in mouse models have shown that ER $\alpha$  and ER $\beta$  have similar yet distinct roles in estrogen action (Couse and Korach 1999; Harris 2007).

Several splice variants of both ER $\alpha$  and ER $\beta$  have been identified but their biological functions are poorly understood (Flourirot et al., 2000; Fujimura et al., 2001; Palmieri et al., 2004; Wang et al., 2005). Among the various splice variants of ER $\beta$ , only the ER $\beta$ 2 isoform (also called ER $\beta$ cx) is best characterized (Fujimura et al., 2001; Palmieri et al., 2004). ER $\beta$ cx differ from ER $\beta$ 1 at the C-terminal end where 61 amino acids that form part of the LBD have been replaced by 26 unique amino acid residues. In vitro assays have shown that ER $\beta$ cx cannot bind ligand or coactivators and is incapable of activating transcription of reporter genes containing ERE sequences. ER $\beta$ cx heterodimerizes with ER $\alpha$  but not with ER $\beta$  and functions as a negative inhibitor of ER $\alpha$  (Ogawa et al., 1998).

Estrogens exert their physiological functions such as growth, development, cellular differentiation and reproduction through the ERs. Estrone (E1), 17 $\beta$ -estradiol (E2) and estriol (E3) are the three major estrogens produced in humans, of which the most predominant and potent form is E2. ERs regulate transcription of genes through different mechanisms. According to the genomic or classical mechanism, E2 binds to ER, followed by dimerization of the receptor and binding to EREs located in the promoter regions of target genes. This results in recruitment of co-activators or co-repressors to the promoter eventually leading to activation or repression of the target gene expression (Nilsson et al., 2001). ERs are also known to regulate transcription of genes that do not contain ERE sequences in their promoter through protein-protein interactions via activator protein transcription factor complex (AP-1) or specificity protein (Sp1) (Paech et al., 1997; Saville et al., 2000). In addition estrogens can also exert their actions rapidly via nongenomic mechanism involving membrane associated ERs or other non-ER plasma membrane associated estrogen-binding proteins (Losel and Wehling 2003). Some of the non-genomic actions of E2 reported in the literature include mobilization of intracellular Ca<sup>2+</sup>, stimulation of adenylate cyclase and regulation of cAMP and activation of kinases (Aronica et al., 1994; Improta-Brears et al., 1999; Migliaccio et al., 1996; Razandi et al., 1999) (Fig 5).



**Fig 5: Schematic drawing of ER signalling mechanisms.** 1. E2-ER complex dimerize and bind to ERE sequences located on the promoters of target genes. 2. E2-ER complex can regulate transcription through protein-protein interactions by tethering to a transcription factor complex (TF) that is bound to the target gene promoter. 3. Ligand-independent activation of ERs at ERE sites through phosphorylation (P). 4. Nongenomic actions of estrogens. These rapid actions of estrogens are mediated by membrane E2-ER complex. Yellow circles: ER, Red circles: E2, White circles : TF. The figure is adapted and modified from the article *Mol endocrinol* (2005) 19(4):833-842.

### 1.5 Estrogen actions in the neuroendocrine system and other areas of the brain

Estrogens are essential for the reproductive functions in females. Estrogens can act at the level of the hypothalamus and the pituitary gland to stimulate the release of gonadotropins and prolactin. Although estrogens are considered as a female hormone, they are also produced in males however at lower amounts and they play an essential role in spermatogenesis (Luconi et al., 2002). In the rat pituitary, lactotropes express the highest levels of ER $\alpha$  followed by the gonadotropes,

corticotropes and somatotropes (Couse and Korach 1999; Mitchner et al., 1998; Scully et al., 1997). ER $\beta$  expression is detected at varying levels in lactotropes, gonadotropes, corticotropes, somatotropes and melanotropes of the rat pituitary however at lower levels than ER $\alpha$ . Only a small fraction of the pituitary cells co-express ER $\alpha$  and ER $\beta$  (Mitchner et al., 1998). Discrepancies in the distribution of ER subtypes within the different cell types of pituitary between different species have been demonstrated by many groups (Mitchner et al., 1998; Wilson et al., 1998).

Characterization of the ER $\alpha$  knock-out mice (ER $\alpha$ KO) have shown that ER $\alpha$  has a significant role in the regulation and secretion of prolactin and gonadotropins. Disruption of ER $\beta$  in mice does not affect the production of prolactin (Couse and Korach 1999; Pelletier et al., 2003; Scully et al., 1997). The gonads which are under control of the hypothalamus-pituitary axis are affected in knock-out models of ER $\alpha$  and ER $\beta$ . In ER $\alpha$ KO female, the sexual maturation of the reproductive tract is disrupted, while in the ER $\beta$ KO female the ovarian function is affected (Couse and Korach 1999). Disruption of the ER $\alpha$  gene in male results in small sized testis, leydig cell hyperplasia in the testis and infertility (Eddy et al., 1996). Phenotype characterization of ER $\beta$ KO male did not show similar testicular defects indicating that fertility of males might be under the control of ER $\alpha$  (Krege et al., 1998).

Localization of ER subtypes in the gonadotropin-releasing hormone neurons have been described by many groups (Hu et al., 2008; Hrabovszky et al., 2000). Hu et al., have shown that ER $\alpha$  is important for the negative regulation of GnRH neuronal function while ER $\beta$  mediates a stimulatory action on GnRH secretion (Hu et al., 2008). The two ER subtypes exhibit selective and differential expression in various neuronal populations in the brain including the hypothalamus. In the GHRH neurons of the rat hypothalamus, only ER $\alpha$  is expressed indicating that estrogens might regulate GHRH through ER $\alpha$  (Kamegai et al., 2001). On the other hand, robust expression of ER $\beta$  mRNA but not ER $\alpha$  in the CRF expressing neurons of the rat PVN has been demonstrated (Laflamme et al., 1998). Estrogens are implicated in the regulation of the HPA axis by stimulating the synthesis of CRF in the hypothalamus (Ochedalski et al., 2007). Exposure to exogenous E2 or ER $\beta$  agonist stimulated the CRF gene transcription. Two ERE half sites have been identified in the promoter of the CRF gene thus clearly indicating that steroid hormones can regulate CRF expression possibly through ER $\beta$  (Chen et al., 2008). Effects of estrogens on the endocrine response of stress have been documented in humans and in rodents. Sex differences in

circulating glucocorticoid levels and in the glucocorticoid response to stress has been reported with females having higher levels than males. In rats it has been demonstrated that estrogen administration causes loss of GR's ability to auto-regulate resulting in functional impairment of the GR (Burgess and Handa 1992; Redei et al., 1994).

Estrogens also control non-reproductive events in the brain. Besides the hypothalamus, cells expressing ERs are also present in other regions of the brain such as the hippocampus, olfactory bulbs, cerebral cortex, midbrain and brain stem (Kuiper et al., 1997; Kuiper et al., 1998b). Estrogen-mediated sexual dimorphism has been noted in extra-hypothalamic structures including basal forebrain, midbrain, brain stem, and structures of the limbic system (McEwen and Alves 1999). Estrogens can regulate the serotonin system which is associated with mood, aggression and cognitive function. ER $\alpha$  and ER $\beta$  subtypes are found in midbrain 5HT neurons of primates and E2 treatment regulates both tryptophan hydroxylase and progesterin receptor (PR) expression (Bethea et al., 1998). In mouse deficient of ER $\alpha$ , estrogen induces the expression of the progesterin receptor implying that ER $\beta$  might regulate these effects (Alves et al., 2000). The role estrogen in cognition has been investigated in humans and in rodents. The hippocampus is associated with memory and steroid hormones have been implicated in the regulation of the formation and breakdown of excitatory synapses in the hippocampus (Woolley et al., 1990). Cholinergic neurons located in the basal forebrain project their axons to the cerebral cortex and hippocampus which are important for cognitive function. Choline acetyltransferase (ChAT) induction in the basal forebrain has been shown following estrogen therapy in ovariectomized female rats with increased ChAT activity in their axon projections (McEwen and Alves 1999). Catecholaminergic neurons of the brain stem consists of small numbers of ER expressing cells which respond to estrogen treatment by altering the levels of tyrosine hydroxylase mRNA (Liaw et al., 1992). Expression of ER $\alpha$  in nor-adrenergic neurons has been reported in rats and sheep (Simonian and Herbison 1997; Simonian et al., 1998). Estrogen regulates dopaminergic neurons (DA) located in the hypothalamic and extra-hypothalamic regions of the brain in controlling movement and behaviour in humans as well as animals. Estrogen action in the DA neurons of the striatum influences the sensorimotor activity in rats (Becker et al., 1987).

Estrogens can influence memory and cognition and offers protection against neurodegenerative diseases like Alzheimer's and Parkinson's. Clinical observations made over the last few years support a positive therapeutic effect of

estrogen in Parkinson's disease (Tsang et al., 2000). A case-control study on older women has suggested that post-menopausal decline in estrogen levels might contribute to the development of Alzheimer's disease and that estrogen replacement therapy might provide beneficial effects by delaying the onset of the disease (Paganini-Hill and Henderson 1994). The neuroprotective effects of estrogen is mediated by many different pathways including ER activation, MAP kinase pathway activation, antioxidant properties of steroids, increased expression of the bcl-2 family proteins and reduction of NMDA receptor activation (Green and Simpkins 2000).

In summary, estrogens which were once believed to have only reproductive functions are now recognized to exert diverse actions throughout the brain. These actions are mediated both by intracellular ERs and membrane bound ER. The neuroprotective effects of estrogen have drawn a lot of interest recently and should be studied in more detail to explore the therapeutic potential of estrogen and estrogen like compounds.

## **1.6 Endocrine disruptors**

Endocrine disrupting chemicals (EDCs) are environmental chemicals that interfere with the normal functioning of the endocrine system causing adverse effects in hormone responsive target cells, tissues and organs. Endocrine disruptors include naturally occurring chemicals such as phytoestrogens and synthetic chemicals like plastics and plasticizers, surfactants, detergents, fungicides, pesticides, industrial solvents and pharmaceutical agents. Endocrine disruption has been reported in humans, rodents, aquatic species and even in the symbiotic relationship between plant roots and bacteria (Arukwe et al., 1997a; Fox et al., 2007; Li et al., 2010; Newbold and McLachlan 1982). EDCs act by multiple signalling pathways. They can bind to nuclear hormone receptors to stimulate or antagonize them, interfere with enzymes involved in steroid biosynthesis, disrupt thyroid hormone transport and action and interfere with neurotransmitter systems. Most of the endocrine disruptors are found to exert estrogenic function. These compounds mimic the function of the endogenous ligand E2 by binding to ER. Some of the well-studied estrogenic endocrine disruptors are ethinylestradiol (EE2), diethylstilbestrol (DES), 4-hydroxytamoxifen, o,p'-DDT, genistein, 4-nonylphenol (4-NP), and bisphenol A. Since DES and 4-NP are the two synthetic estrogen mimics/EDCs used in paper II & III, a brief summary about these compounds and their endocrine disrupting actions are presented below.

### 1.6.1 Diethylstilbestrol

Diethylstilbestrol is a nonsteroidal pharmaceutical agent prescribed in the late 1940's to pregnant women to prevent miscarriages and premature birth. However, after more than two decades, it was reported that DES caused a rare cancer termed vaginal clear cell adenocarcinoma in daughters of the women who had taken DES during their pregnancies (Herbst et al., 1971). Follow up studies further reported adverse anatomical malformations in both male and female offsprings due to neonatal exposure of DES (Herbst 2000; Kaufman et al., 2000). DES treatment in mouse models show higher incidence of reproductive tumors and reproduced many of the morphological defects that were observed in DES exposed humans (Newbold 2004). ER knockout mice were helpful in elucidating the mechanism of action of DES. Results from DES treatment of wildtype, ER $\alpha$ KO and ER $\beta$ KO mice suggest that DES elicits its deleterious effects via ER $\alpha$  in both sexes (Couse et al., 2001; Couse and Korach 2004; Prins et al., 2001). Genes from the *Hox* and *Wnt* families have been implicated in DES induced changes in the reproductive tissues. Female mice treated with DES *in utero* show decreased expression of *HoxA9* in the oviduct and *HoxA10*, *HoxA11* in the uterus (Block et al., 2000). Down-regulation of the *Wnt7a* mRNA has been demonstrated in the uterus of female mice following exposure to DES (Miller et al., 1998). Furthermore, results from the studies in ER $\alpha$ KO mice support the theory that alterations in *Hox* and *Wnt* gene expression following DES treatment may be in part mediated through ER $\alpha$  (Couse et al., 2001).

### 1.6.2 4-nonylphenol

Nonylphenol ethoxylates (NPE) are used in the production of nonionic surfactants which are widely used in cleaning agents, plastic industries, textile and paper industries (Liber et al., 1999). 4-nonylphenol, an endocrine disruptor is produced by microbial degradation of NPE during sewage treatment and is released in high amounts into the environment from industrial and sewage treatment plants (Giger et al., 1984; Nimrod and Benson 1996). Bioaccumulation of 4-NP in microorganisms, plants and fish have been reported (Ahel et al., 1993; Bokern et al., 1998; Karley et al., 1997). It has been shown previously that 4-NP is capable of mimicking the actions of E2 by binding to ERs and inducing the synthesis of hepatic vitellogenin protein (VTG) and zona radiata protein (ZRP) (Arukwe et al., 1997b; Madsen et al., 1997; White et al., 1994; Yadetie et al., 1999). Studies using cell lines have showed that the binding

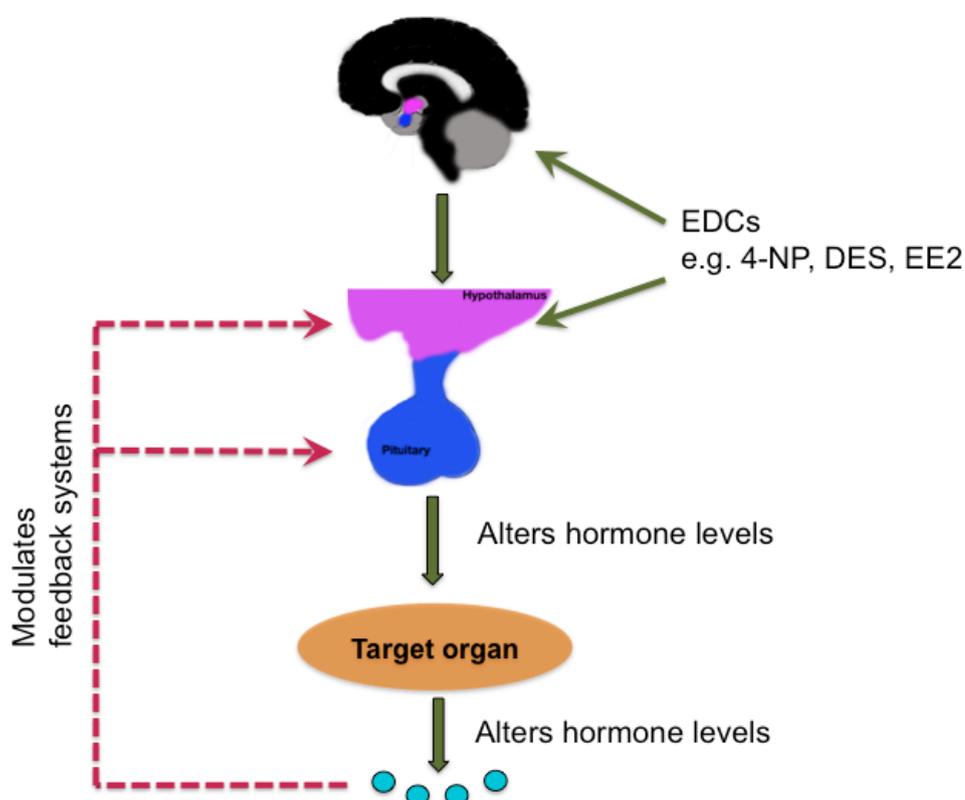
efficiency of 4-NP is 1000-10,000 fold less than E2 (Nimrod and Benson 1996). In rodent models it was shown that 4-NP could induce uterine growth in females similar to E2 despite its weaker estrogenic potency (Lee and Lee 1996).

Effects of 4-NP on reproduction has been studied extensively in varied fish species. Induction of VTG in males and the occurrence of testis-ova are generally used as endpoints to assess the toxicity of 4-NP on reproduction. Recently it has been shown in zebrafish, that 4-NP at concentrations lower than what is reported for other fish models had the capacity to induce *vtg* expression in males and affect reproduction in females (Yang et al., 2006). Aromatase which is responsible for the conversion of androgens to estrogens is known to be modulated by many EDCs including 4-NP. Transcription of the brain isoform of aromatase, *cyp19a2* is increased when zebrafish juveniles were treated with ecologically relevant concentration of 4-NP for a period of 3 days (Kazeto et al., 2004). Maeng et al have reported the pituitary responses of 4-NP in masu salmon. They show that 4-NP treatment in female juvenile masu salmon induces the expression levels of GTH $\alpha$  and LH $\beta$  possibly in an ER $\alpha$ -dependent pathway (Maeng et al., 2005). In juvenile Atlantic salmon it has been shown that both E2 and 4-NP induce LH $\beta$  mRNA levels in females suggesting that 4-NP can mimic E2 and perturb the regulation of LH $\beta$  at the level of the pituitary (Yadetic and Male 2002). The effects of 4-NP on the hypothalamus has been demonstrated in rainbow trout. The results of the study show that 4-NP decreased *GnRH2* gene expression in a dose dependent manner (Vetillard and Bailhache 2006). Gene expression profiling of liver samples from adult male zebrafish has shown that 4-NP and E2 are capable of activating the expression of some genes in a different manner indicating that 4-NP has the capacity to act via an alternative pathway different from the traditional estrogen mediated pathway (Ruggeri et al., 2008). In paper III we have demonstrated that this compound can affect the notochord, the muscle function and the neuroendocrine system in zebrafish embryos and larvae possibly involving several pathways.

Due to the wide-spread use of this compound, the chances of human exposure to 4-NP are very high. Studies on a demographically diverse population in the United States has reported the presence of 4-NP in urine samples (Calafat et al., 2005). Although numerous studies report the adverse effects of 4-NP in a wide range of species, its potential effects on the human population is still unknown. Hence, studies are warranted in developing methods to identify 4-NP and its metabolites in humans and in understanding the harmful health effects caused by 4-NP in the human population.

## 1.7 Effects of EDCs on the neuroendocrine system

The hypothalamus and the pituitary gland, the central unit of the endocrine system are the important targets of estrogens and EDCs. Besides the neuroendocrine system, estrogens can also act on other brain areas such the serotonergic system, catecholaminergic neurons, the forebrain cholinergic system and hippocampus. This explains that in addition to reproductive functions, estrogens are also important for non-reproduction functions such as learning and memory, emotions, motor co-ordination and sensitivity to pain. Since the major focus of this thesis is the neuroendocrine system, the effects of endocrine disruptors on some of neuroendocrine axes are discussed (Fig 6).



**Fig 6: Schematic representation of the neuroendocrine targets of EDCs.**

*EDCs can target the neurotransmitter systems of the brain or the hypothalamus or the pituitary gland to modulate the synthesis of hormones of the hypothalamus and pituitary. This alters the production of hormones from the target endocrine organs. The hypothalamus, pituitary and endocrine glands interact with each other and therefore alteration of hormone levels at any one of the sites can totally disrupt the normal functioning of the neuroendocrine system.*

### 1.7.1 The HPG axis

There is an accumulation of data on the effects of EDCs on reproduction. For proper reproductive function, the hypothalamic-pituitary-gonadal axis and other organs that are involved in reproduction and are regulated by steroids (e.g. uterus and mammary gland) should function normally. Most EDCs affecting reproduction possess estrogenic or antiestrogenic activity. They are known to mediate their deleterious effects by binding to ER or androgen receptor (AR). *In vitro* studies using the GnRHGT1-7 cell line has shown that polychlorinated biphenyls can alter GnRH expression in these cells. It is important to note that these cells express ER $\alpha$  and ER $\beta$  and that the effects of PCB are blocked by the estrogen antagonist ICI 182,780 suggesting that ERs could be involved in this mechanism (Gore 2002). Studies in rats have also shown that several estrogen mimics such as bisphenol A, o,p' DDT have an impact on the release of GnRH leading to dysregulation of the HPG axis (Rasier et al., 2008). The effects of PCB was also shown in the Atlantic croaker where it decreased the content of GnRH in the preoptic area of the hypothalamus. In addition, a reduction in the number of GnRH receptors and a significant decrease in LH production have been noted in the pituitary (Khan et al., 2001).

A considerable number of studies have provided evidence for impaired reproduction caused by exposure to estrogenic compounds. Daughters of women exposed to DES show severe effects with a T-shaped uterus, infertility, spontaneous abortion, ectopic pregnancy and preterm delivery (Kaufman et al., 2000; Schragger and Potter 2004). Genistein which is a member of the phytoestrogen family has been linked to infertility in animal models (Setchell et al., 1987). Furthermore, in a neonatal mouse model it has been shown that genistein could induce uterine adenocarcinomas (Newbold et al., 2001). Studies using ER $\alpha$ KO and ER $\beta$ KO show that genistein can signal through both ER subtypes to mediate its estrogenic effects however in a tissue specific manner (Jefferson et al., 2002; Klotz et al., 2000). Estrogenic compounds have been shown in various species to also affect the male reproductive tissues. Experiments with neonatal mouse model has demonstrated that DES treatment decreases seminal vesicle size and induces several abnormalities in the prostate gland (Prins et al., 2001). Guppies treated with octylphenol and E2 for 30 to 60 days displayed decreased testis growth accompanied by decrease in male secondary sexual characteristics (Toft and Baatrup 2001). The time of development during which an organism is exposed to endocrine disruptors appears to be critical. For example, medaka exposed to

octylphenol at 3 days post hatch showed highest incidence of intersex than when exposure occurred at later time points (Gray et al., 1999).

### **1.7.2 The thyroid hormone axis**

Extensive studies on EDCs have shown that the hypothalamic-pituitary-thyroid axis is also sensitive to endocrine disruptors present in the environment. Acute exposures to PCB congeners disrupt the normal function of the HPT axis in developing rats by reducing the response of the pituitary and thyroid to TRH stimulation (Khan and Hansen 2003). PCB is also known to affect behaviour and cerebellum development differentially in male and female rat neonates with greater influence on the male rats (Nguon et al., 2005). Bisphenol A generally considered as an estrogen can interfere with thyroid function by antagonizing thyroid hormone receptor  $\beta$  ( $\beta$ -TR) and elevating the levels of T4 in rats (Zoeller et al., 2005). A wide variety of endocrine disruptors including PCB have been shown to dysregulate the thyroid axis also in fishes. The mechanisms by which PCB and bisphenol-A act on the thyroid axis is still unclear. Whether ER-mediated signalling is involved in their mode of actions on the HPT axis needs to be addressed.

Proper thyroid function is essential for the maintenance of energy balance and metabolism in the body. Therefore, disruption of the HPT axis could lead to adverse health problems like obesity. Numerous research findings have associated PCB, Bisphenol-A and DES with obesity. The mechanism by which these chemicals act in promoting obesity is poorly understood.

### **1.7.3 The HPA axis**

Previous studies have demonstrated that estrogens participate in the release of ACTH and cortisol/corticosterone during stress. Phytoestrogens have been shown to activate the HPA axis by increasing GR levels in the hippocampus. This study explains that life-long consumption of dietary phytoestrogens (soy-derived phytoestrogens) can alter the stress response in male rats to a female-like pattern (Lephart et al., 2003). Chronic exposure of goldfish to 4-NP did not alter plasma cortisol levels suggesting impairment in the responsiveness of goldfish to environmental stressors like 4-NP, which might be due to the prolonged hyperactivity of the cortisol-producing endocrine system (Palermo et al., 2008).

There is limited information about the toxic effects of endocrine disrupting chemicals on the adrenal function. From the above mentioned examples it is obvious

that EDCs can disrupt the HPA axis which is a serious concern. Future research should focus on investigating specific effects of EDCs on the stress axis.

#### **1.7.4 The growth hormone axis**

Growth hormone (GH) is produced by the pituitary gland and its synthesis is controlled by hormones produced in the hypothalamus. GH is important for growth, osmoregulation, metabolism, reproduction and development. Many previous studies have described that secretion and action of GH are modulated by E2 and estrogen mimicking compounds. Therefore, accumulation of estrogenic compounds in our environment can affect the hypothalamus-pituitary function by affecting the synthesis, release and physiological function of GH.

Effects of estrogen mimics on the secretion of GH have been well-studied in several vertebrate models. For example, rainbow trout pituitary cells exposed to o,p'-DDT and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) showed increase in the mRNA levels of GH through the direct estrogenic pathway for the former and ER/AhR cross talk for the latter (Elango et al., 2006). Bisphenol-A, has been shown to modulate the somatostatinergic system in the diencephalic area of *Coris julis* which might in turn alter the levels of pituitary GH by disrupting the inhibitory action of somatostatin on GH synthesis (Alo et al., 2005). Dysregulation of GH gene expression by EDCs in mammals have also been reported in many studies. Phenolic xenoestrogens like octylphenol (OP), nonylphenol (NP) and bisphenol-A have been reported to modulate GH gene expression in rat pituitary GH3 cells by both genomic and non-genomic pathways (Dang et al., 2009).

Estrogens can not only act at the level of the brain and pituitary but can also interrupt the metabolic action of GH by targeting the liver especially the GH/IGF-I axis. Humans are continuously exposed to estrogen-like compounds during their life time. In addition, estrogens are also widely used as therapeutic substances. The side effects of oral estrogens and estrogen-like compounds on the metabolic action of GH in liver have been extensively described (reviewed in (Leung et al., 2004).

#### **1.8 Estrogen receptors in fish**

Similar to mammals, fishes possess alpha and beta subtypes of ER. ER $\alpha$  was first identified in the rainbow trout (Pakdel et al., 1989). The rainbow trout has two alpha subtypes, ER $\alpha$ 1 and ER $\alpha$ 2 but it is not clear whether they are derived from two distinct genes (Nagler et al., 2007). In addition, the existence of two isoforms of ER $\alpha$

derived from alternative splicing has also been reported (Pakdel et al., 2000). The long isoform of rtER $\alpha$ L shares higher percentage of homology with mammals and exhibits ligand-dependent transcriptional activity whereas the short isoform rtER $\alpha$ S is truncated by 45 residues at the N-terminal region and has ligand-independent transcriptional activity representing i.e. 30% of the total receptor activity (Pakdel et al., 1990; Pakdel et al., 2000). The long isoform is shown to be expressed in a wide range of tissues such as the liver, brain, pituitary and ovary whereas the short form is expressed only in the liver and might have a role in the production of vitellogenin in the liver (Menuet et al., 2001).

ER $\alpha$  has also been identified and characterized in other fish species such as tilapia, gold fish, zebrafish, channel catfish, japanese eel, red sea bream and gilthead sea bream (Choi and Habibi 2003; Meneut et al., 2002; Munoz-Cueto et al., 1999; Tan et al., 1995; Todo et al., 1996; Xia et al., 1999). In teleosts, there are two subtypes of ER $\beta$  which are derived from two distinct genes. The two beta forms appear to have arisen by gene duplication. Numerous studies carried out in different fish species show that these two forms exhibit differential and tissue-specific expression (Choi and Habibi 2003; Hawkins et al., 2005; Meneut et al., 2002; Pinto et al., 2006; Sabo-Attwood et al., 2004). Recently in the Atlantic croaker, a membrane bound G-protein-coupled estrogen receptor (GPER or GPR30) has been identified in the gonads and it is possible that it regulates the non-genomic actions of E2 as demonstrated in mammals (Thomas et al., 2006). Inhibitory actions of estrogens on zebrafish oocyte maturation has been studied very recently and the outcome of this study reveals that estrogen actions are mediated through GPER (Pang and Thomas).

### **1.9 Zebrafish as a model organism**

Zebrafish is a popular teleost model to study developmental biology and genetics. Zebrafish and humans display similarities which includes developmental process, genes, anatomy, and physiology. Zebrafish is easy to maintain and raise as they require limited space owing to their small size. Another important advantage in using zebrafish is the sample size. Each female can lay approximately 200-300 eggs which develop rapidly, completing embryogenesis in just three days. As the embryos are transparent, morphological structures and organs can be visualized under a dissection microscope. Moreover, optical clarity allows for direct visualization of phenotypic changes during mutant screening, morpholino knockdown experiments,

screening of environmental pollutants for toxicity, drug screening etc. In addition they become sexually mature within 3-4 months which facilitates the development of transgenic fish, mutants, tests drugs and assess toxicity of environmental pollutants such as endocrine disruptors in adult tissues.

Large-scale genetic screens for mutations in zebrafish carried out about 15 years ago resulted in the identification of several mutations in genes that affect numerous developmental processes including morphogenesis, organogenesis and differentiation (Driever et al., 1996; Haffter et al., 1996). Many of the so mutated genes in zebrafish are found to be orthologs of mammalian genes and they produce phenotypes similar to that observed in humans. Therefore, zebrafish mutants will be very useful for studying various human diseases. Over the last few years, new genetic methods have been developed to generate mutations in genes. Specific genes are mutated using reverse genetics strategies known as TILLING and insertional mutagenesis using retroviral vectors (Amsterdam et al., 1999; Wienholds et al., 2002)). Very recently, targeted mutations in the zebrafish germ line has been achieved using zinc finger nucleases (Meng et al., 2008). An alternative approach to determine the function of a gene in early embryonic development is to knockdown the target gene using morpholino antisense oligonucleotides. However, it is very important to standardize the concentration of the morpholino that can effectively block translation without causing any off-target effects. These well-established technologies provide excellent tools to identify and determine the function of genes that are required for the development and function of the neuroendocrine system. Additionally, zebrafish mutants can be used to decipher the mode of action of toxic chemicals.

### **1.9.1 The zebrafish HPI axis development**

The hypothalamus and the pituitary gland form the major link to the endocrine organs. Currently various groups are investigating the ontogeny of hypothalamic and pituitary cell types in zebrafish. Also greater efforts are put forth in identifying genes that may play an important role in the specification of these neuroendocrine cell types. To some extent the development of the pituitary gland has been well described (Pogoda and Hammerschmidt 2009). On the contrary the hypothalamus development in zebrafish has received less attention. Through knock-out mouse models, the homeobox genes *Orthopedia* and *Brn2* and the bHLS-PAS genes *Sim1* and *Arnt2* have been identified to be essential for the development of CRH neurons (Acampora et al., 1999; Keith et al., 2001; Michaud et al., 1998; Nakai et al., 1995; Schonemann et al., 1995)).

Whether these transcription factors have similar roles in controlling CRH cell development in zebrafish remains to be explored. This information will be very valuable in understanding not only the ontogeny of the CRH cell lineage but also the establishment of the HPI axis during embryonic development of zebrafish.

The HPI axis controls stress responses in zebrafish by stimulating the release of CRH in the hypothalamus which subsequently activates the pituitary corticotropes to synthesize ACTH which in turn stimulates the interrenal cells to produce and secrete cortisol into the circulation. As in humans, cortisol is the stress response hormone in zebrafish which makes zebrafish a suitable model to study stress physiology. Little is known about the development of the HPI system in zebrafish. We have taken the initiative to describe the *crh* gene expression in zebrafish embryos (paper I). Characterization of CRH and CRH related peptides is the first step towards the understanding of the molecular programming in the development of the stress axis in zebrafish. Transcript distribution of urotensin 1 and 3 (*uts1* and *uts3*), which are the members of the corticotropin releasing hormone family have been described recently in the embryonic brain of zebrafish (Brautigam et al., 2010).

In the pituitary, ACTH is produced by the cleavage of the large precursor protein proopiomelanocortin (POMC). In zebrafish, POMC is first detected in the pituitary around 24 hpf. The anterior POMC domain corresponds to the corticotrophic cells that synthesize ACTH (Liu et al., 2003). The zebrafish *eyal* gene has been shown to be required for the differentiation of corticotropes, melanotropes and gonadotropes and the *aal* mutant carrying mutation in *eyal* lack these three cell types (Nica et al., 2006). Mutations in the zebrafish *six1* gene show a similar but weaker phenotype compared to the *eyal/aal* mutants with *pomc* expressing cortico- and melanotropes being severely affected (Pogoda and Hammerschmidt 2009). Another zebrafish mutant, *lia* that harbors mutation in the *fgf3* gene affects the specification of all cell types of the adenohypophysis including corticotropes (Herzog et al., 2004). Thus far, *fgf3*, *eyal* and *six1* appear to be essential for the corticotrope cell differentiation/specification.

Previous studies have shown that the development of the interrenal organ in zebrafish is similar to the adrenal gland development in other vertebrate models. The nuclear receptor, *fflb*, the homolog of the mammalian steroidogenic factor (SF-1) is essential for the differentiation of interrenal cells in zebrafish and it stimulates the side chain cleavage of cytochrome P450 (Hsu et al., 2003). The organogenesis of the zebrafish interrenal organ has been studied elaborately by To et al. Until 2 dpf the development of the interrenal organ is not under the control of the pituitary. However,

by 5 dpf, inputs from the pituitary corticotrophs are needed for sustaining the interrenal function and this is mediated through the Mc2 receptors (To et al., 2007).

Cortisol levels in zebrafish embryos increase after 42 hpf and reaches peak levels by 6 dpf when most of the genes involved in the glucocorticoid synthesis pathway are expressed (Alsop and Vijayan 2008). The first cortisol response to stress has been observed in early larval stages around 97 hpf. The late cortisol stress response despite the early expression of *crh* (25 hpf) and *acth* (48 hpf) genes is intriguing. Therefore, it becomes important to elucidate the molecular events involved in initiation of the stress response in zebrafish. The already available zebrafish mutants lacking corticotropes may throw some light on the onset of the HPI axis activation in zebrafish.

### **1.9.2 Estrogen receptor subtypes in zebrafish**

There are three forms of ERs in zebrafish which are derived from three distinct genes. They are termed *esr1* (ERalpha; zfER $\alpha$ ), *esr2b* (ERbeta1; zfER $\beta$ 1), and *esr2a* (ERbeta2; zfER $\beta$ 2) (Bardet et al., 2002; Meneut et al., 2002). Sequence homology comparison and phylogenetic analysis of zebrafish *esr* genes between ERs of mammals and fish species revealed that the two zfERbeta subtypes arose from duplication of the ER $\beta$  gene. We and others have described the developmental expression pattern of the three ER forms in zebrafish. These studies have shown that all three ER forms are maternally derived and that the expression of *esr1* and *esr2b* progressively increased from 24 hpf. The maternal expression of *esr2a* diminished after mid blastula transcription and the expression of the gene appears later during larval stages ((Bardet et al., 2002; Chandrasekar et al., 2010; Mouriec et al., 2009; Tingaud-Sequeira et al., 2004)). High levels of *esr2a* and *esr2b* and low levels of *esr1* have been detected in the epidermis, pectoral fin buds, hatching gland and in brain at 24 hpf. Both *esr2a* and *esr2b* are also found to be present in primary neuromast cells (Tingaud-Sequeira et al., 2004). In adult tissues, the ERs are highly expressed in the liver, brain and testis (Chandrasekar et al., 2010; Legler et al., 2000; Meneut et al., 2002). Differences in the mRNA expression of the *esr* genes in various tissues have been reported recently (Chandrasekar et al., 2010). Characterization of zfERs mRNA expression in adult brain has shown that the three ERs exhibit differential and partly overlapping distribution in the preoptic area and the mediobasal hypothalamus providing evidence for the importance of ERs in the neuroendocrine function (Meneut et al., 2002).

The distribution of *esr* genes in a wide range of embryonic and adult tissues suggest that estrogens may have non-reproductive functions in zebrafish as observed in mammals. Knockdown of *esr2a* (*erβ2*) in zebrafish embryos disrupted the development of neuromasts in zebrafish suggesting that estrogens and their receptors may have a vital role in the development of the mechano-sensory system. It was further shown in this study that *erβ2* promote hair cell differentiation by interacting with notch signalling molecules (Froehlicher et al., 2009). The zebrafish *esr1* gene has been implicated in controlling the migration of the primordial cells of the posterior lateral line system (Gamba et al., 2010). Morpholino knockdown of *esr2b* has not been reported so far. An elucidation of *esr2b* function is needed to get an overview on the roles of ERs in embryonic development of zebrafish.

Through binding assays it has been shown that the zfER proteins exhibit high affinity for E2 and zfERβ2 is found to be more sensitive to E2 than the other two zfERs. Computational homology modelling of the zfERs revealed that the E2-binding sites in zfERs are identical to their respective human homologs and fluorescently tagged E2 could bind to zfERα in a manner similar to human ERα suggesting that an *in silico* docking study could be helpful in predicting the endocrine disrupting activity of environmental pollutants (Costache et al., 2005). Exposure to exogenous E2 has shown that ERs of zebrafish can be regulated in a tissue specific manner (Chandrasekar et al., 2010; Meneut et al., 2002; Meneut et al., 2004). To facilitate large scale screening to identify estrogenic chemicals in *in vivo* systems, transgenic zebrafish expressing estrogen responsive reporter genes have been developed. For example, using transgenic zebrafish expressing the luciferase reporter gene under control of ERE sequences, the period of gonad differentiation has been determined to be the most sensitive developmental stage for exposure to E2 and the reproductive organs appear to be the major target for xenoestrogens (Legler et al., 2000).

### **1.9.3 Assessment of toxicity and endocrine disruption using zebrafish**

Zebrafish have been used for many years to test the toxicity of synthetic chemicals, natural products, metals and organic solvents. Both adult fish and embryos are used for screening chemical toxicants. Embryos are highly sensitive to environmental toxicants and are used to identify chemicals that may affect developmental processes such as organogenesis. Developmental effects of (TCDD) have been thoroughly studied using zebrafish. Morpholino knockdown technology

enabled researchers to elucidate the mode of action of TCDD in zebrafish. The findings show that TCDD exerts its toxic effects through the aryl hydrocarbon receptor (AHR) and zfARH1 is found to be involved in mediating these effects (Prasch et al., 2003).

Induction of VTG in males is considered as a reliable endpoint for the assessment of endocrine disruptors. Histopathology is frequently used to monitor tissue- and cell type specific alterations caused by EDCs. Several transgenic lines have been developed for fast and reliable screening of EDCs. Zebrafish microarray chips are commercially available and can be used to determine genome wide gene expression changes caused by EDCs. Since zebrafish have a short life cycle, chronic effects of estrogenic compounds can be easily studied. Life cycle exposure of EE2 induced vtg synthesis, delayed onset of sexual maturation, altered sex differentiation and sex ratio and reduced fecundity and fertilization success in zebrafish (Schafers et al., 2007). Zebrafish is also well suited for studying toxic mechanisms of EDCs because it shares a high degree of similarity in hormone signalling pathways with other vertebrate models.

Although numerous data on endocrine disruption are available in the literature, the use of zebrafish in studies related to endocrine disruption is still in its infancy. This is because many of the EDCs affect gonad development and reproduction in zebrafish and the genetic pathways involved in zebrafish sex determination and differentiation are not yet understood. Thus the lack of knowledge stand in the way of understanding how EDCs derail the reproductive function in zebrafish. Perhaps zebrafish mutants with defects in gonad development might answer some of the questions pertaining to zebrafish sex determination and EDCs role in modulating reproduction. I hope that the continued technical advances in zebrafish will attract more and more toxicologists and endocrinologists to use this model in the future to study endocrine disruption.

## 2. AIMS

This thesis is focused on three different aspects in connection with the neuroendocrine system controlling stress and reproduction. As the neuroendocrine system in zebrafish is similar to humans, this model is suitable to study development, function and disruption of the neuroendocrine system. Moreover, zebrafish is simpler than other animal models to assess the developmental toxicity of EDCs.

The development of the HPI axis in fish, including zebrafish is not well defined. It is only during the last few years that the developmental expression patterns of some of the genes encoding proteins crucial for the functioning of the HPI axis have been described. There is sparse information on the development of the hypothalamic CRH neurons. As a first step towards understanding the development of the neuroendocrine stress axis we have characterized the embryonic expression of the *crh* gene.

The estrogens, produced by the gonads, act on the hypothalamus and the pituitary gland to modulate the reproductive axis. The biological effects of estrogens are mediated by ERs and a plethora of genes have been identified to be under the regulatory control of ERs. However little is known about the regulation of ER subtypes by estrogen mimics, especially in teleosts.

The developmental toxicity of one of the popular estrogen mimics, 4-NP has not been studied in detail, therefore, we have characterized the morphological defects induced by 4-NP in zebrafish embryos and larvae.

Specific aims:

1. To characterize the expression pattern of the *crh* gene in the embryonic zebrafish brain (paper I)
2. To analyze the mRNA levels of ER subtypes in embryos, larvae and adult tissues in the absence and presence of exogenous ligands (paper II)
3. To investigate the developmental toxicity of 4-NP in zebrafish embryos and larvae (Paper III)

### **3. MATERIALS AND METHODS**

#### **In situ hybridization (ISH)**

ISH is a commonly used technique to characterize the expression patterns of genes in tissues or cells. Specific RNA or DNA sequences in tissue sections, whole tissues (whole mount e.g. zebrafish embryos) or cells can be detected by hybridizing the samples with labeled complementary RNA or DNA sequences (probes). The probes can be labeled with radioisotope or fluorescent or non fluorescent agents (e.g. digoxigenin) and can be detected by autoradiography or fluorescent microscopy or color reaction. We have used both fluorescent and digoxigenin probes (paper I & III). RNA probes labelled with digoxigenin were detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase which was later visualized using BCIP/NBT or fast red substrate. Multiple transcripts in zebrafish embryos were detected with two color in situ hybridization using digoxigenin and fluorescent labelled probes simultaneously (Paper I). ISH was used in paper I to determine the spatial expression pattern of *crh*. In paper III, this technique was used to examine the phenotypic changes induced by 4-NP in developing tissues of zebrafish embryos by analyzing the expression patterns of certain marker genes.

#### **Immunohistochemistry (IHC)**

IHC is extensively used to localize proteins in cells of tissue samples. Briefly, an antibody (primary antibody) that can specifically bind to the target antigen in the tissue is used. The primary antibody is then recognized by a secondary antibody tagged to fluorophore such as FITC or non-fluorescent agents (e.g. alkaline phosphatase) which can be visualized by fluorescent microscopy or a color reaction. Axonal tracts, actin filaments and primary motor neurons in zebrafish embryos were detected by IHC (paper I and III).

#### **Real-time PCR**

Real-time PCR (qPCR) is a powerful method used to quantitate mRNA and DNA. It has a wide range of applications including gene expression analysis. It is an efficient, sensitive, accurate and reliable technique. Conventional PCR is a semi-quantitative method where accumulation of the product is measured at the end of PCR reaction making it difficult to determine the concentration of the starting material.

Whereas qPCR allows us to measure PCR amplification as the reaction proceeds. Quantification is performed at the exponential phase which is more precise than the end point assessment in conventional PCR.

qPCR is a fluorescent based method in which the amplicon can be detected either using dsDNA-intercalating agents such as SYBR Green I or hydrolysis probe (e.g. TaqMan probes). SYBR Green I is a dye that emits fluorescence when bound to double stranded DNA (amplicon). As the DNA is amplified, the fluorescent signal will increase and the intensity of the signal corresponds to the amount of amplicon produced in the PCR reaction. The major limitation with the SYBR Green I chemistry is that it can also detect non-specific products due to the presence of any dsDNA in the reaction mixture.

TaqMan probe assay is another commonly used qPCR assay which has a high specificity. The taqMan probe has a fluorescent reporter dye at the 5' end and a quencher at the 3' end. During the PCR reaction the probe anneals between primer sites and is cleaved by the 5' nuclease activity of Taq polymerase. As a result of the cleavage the reporter dye in the probe is separated from the quencher resulting in an increase in fluorescence emission. The increase in the signal is directly proportional to the amount of PCR product formed.

We have used conventional PCR to describe the tissue distribution and developmental expression of *crh* gene in paper I. A SYBR Green I based qPCR assay was employed to measure the relative expression levels of *esr* genes, *crh* and *lhβ* in papers II & III.

### **Calcein staining**

Calcein staining is used to visualize skeletal structures in live zebrafish. It is a simple, sensitive and rapid procedure. Calcein is a fluorescent dye that binds to calcium in bone. Owing to its simplicity, this staining procedure is widely used in the screening of zebrafish skeletal mutants.

### **Mechanical analysis of the muscle of zebrafish larvae.**

A novel method to analyze the mechanical function of zebrafish larval muscles was first described by Dou et al (Dou et al., 2008). In summary, 5 day old zebrafish larvae were anesthetized with 0.017% tricaine and mounted on a set up using aluminium clips to hold the head and the tail of the larvae. The set up was placed in a bath perfused with Krebs-Henseleit solution at 22°C. Holes were made in the

aluminium foil in order to hold the larvae firmly between a pin and an extended arm of the transducer. The length of the preparation can be adjusted with the help of a screw on the pin allowing us to stretch the larval muscles. The bath is placed over an inverted microscope to observe muscle structure. The muscles were stimulated via platinum electrodes and the force signal was recorded using an AD-converter. An illustration of the zebrafish larvae preparation for mechanical analysis can be found in Dou et al (Dou et al., 2008). This set up was used to analyze the muscle function of the larval muscles of control (0.2% DMSO) and 4-NP (2  $\mu$ M) treated zebrafish embryos (paper III).

### **Transmission electron microscopy**

Transmission electron microscopy is a microscopic technique used to observe the ultrastructure of biological and material specimens. Unlike the regular light microscope where light is transmitted through the samples, in TEM a beam of electrons are transmitted through specimens to obtain an image of very high resolution. Minute details of the specimens can be achieved using TEM. We have employed TEM to observe the ultrastructure of the notochord in zebrafish embryos (paper III).

### **The TUNEL assay**

We used the In Situ Cell Death Detection Kit from Roche to detect apoptotic cell death in zebrafish embryos treated with 4-NP. This is a very simple, fast and non-radioactive technique. DNA fragmentation occurs during apoptosis resulting in free 3'-OH ends which can be detected with labeled nucleotides in an enzymatic reaction. The kit contains the TdT (Terminal deoxynucleotidyl transferase) enzyme that will catalyze the polymerization of fluorescein labeled dUTP to 3'-OH DNA ends. Apoptotic cells can be detected either directly by fluorescent microscopy or visualized by a color reaction using an anti-fluorescein-alkaline phosphatase antibody and BCIP/NBT substrate.

## 4. RESULTS AND DISCUSSION

We have used zebrafish as a vertebrate model to understand the development, function and disruption of the neuroendocrine system. Furthermore we have analyzed the developmental toxicity of 4-nonylphenol, a well known endocrine disruptor.

### **Paper I: Characterization of *corticotropin-releasing hormone* in zebrafish embryonic brain**

Corticotropin releasing hormone plays a major role in maintaining the homeostasis of the stress axis (HPG/HPI). With the aim to understand the ontogeny of the CRH cell lineage we analyzed the distribution of *crh* transcripts in the developing brain of zebrafish. The gene sequence of the zebrafish *crh* gene was identified from the zebrafish genome sequence database using bioinformatic tools. We used this information to probe the EST database and identified an EST containing the complete preproCRH open reading frame. The total length of the cDNA was 1,095 bp including a 150 bp 5' untranslated region (UTR), a 489 bp ORF and a 439 bp 3'UTR. The preproCRH open reading frame contained a signal peptide at the N-terminal end, a cryptic peptide and the mature peptide at the C-terminus. Sequence comparison of the mature peptide with other teleosts revealed that the zebrafish mature peptide sequence was identical to goldfish and carp. Using non-quantitative RT-PCR we examined the developmental expression pattern and tissue distribution of *crh* mRNA during different developmental stages and in adult tissues. The expression of *crh* transcripts were detected from 1 dpf onwards and in adult tissues *crh* was expressed in the brain, eye, gills and testis.

By one and two-color in situ hybridization and immunohistochemistry we analyzed the spatial distribution of *crh* in the embryonic brain of zebrafish. In the ventral telencephalon, one or two *crh* expressing cells were detected at 28 hpf which later increased in number and appeared as bilateral clusters at 36 hpf. In comparison to the expression domains of *pax6.1* and *dlx2*, we identified the *crh* transcripts to be located in the subpallium and in the pallium. In zebrafish, the expression of *isotocin-neurophysin (itnp)* marks the preoptic area of the hypothalamus (Unger and Glasgow 2003). Two-color in situ hybridization with *itnp* revealed that the *crh* transcripts were localized within the *itnp* expression domain in the preoptic area. In teleosts, the neurohypophyseal peptides, *itnp* and arginine vasotocin-neurophysin (*vtn*) are synthesized in the dorsal preoptic region of the hypothalamus and they are released into

the pituitary gland through blood vessels (Acher et al., 1997; Venkatesh and Brenner 1995). Hence the expression of *crh* in the preoptic area is consistent with its function as a hypophysiotropin. In adult teleosts CRH is synthesized in the nucleus preopticus (NPO). Co-distribution of CRH, isotocin, and vasotocin in the NPO have been demonstrated in some adult teleosts brain (Huisling et al., 2004; Olivereau et al., 1988; Olivereau and Olivereau 1988; Pepels et al., 2002; Pepels and Balm 2004; Yulis et al., 1986; Yulis and Lederis 1987; Zupanc et al., 1999). Our results suggest that in zebrafish, the preoptic region expressing *crh* mRNA could correspond to the developing NPO.

In the diencephalon, *crh* expression was localized in the posterior tuberculum and in the thalamus. Additionally, few cells positive for *crh* expression were also detected in the midbrain tegmentum. CRH immunoreactivity in similar regions have been reported in other teleost models such as goldfish and flounder (Bernier et al., 1999; Lu et al., 2004).

In zebrafish, the forebrain patterning genes *pax6.1*, *dlx2* and *shh* were used to describe the prosencephalic subdivisions with respect to the prosomeric forebrain model proposed by Puelles and Rubenstein (Puelles and Rubenstein 1993, 2003). Based on the prosomeric model, zebrafish diencephalon is subdivided into three prosomeres p1, p2 and p3. In relation to this model, the pretectum, the thalamus and the prethalamus correspond to the alar p1-p3 respectively. In order to map the precise position of the *crh* expression domains within the forebrain we performed two-color in situ hybridization with *pax6.1*, *dlx2* and *shh*. With respect to the prosomeric model, we proposed that the subpallial and preoptic *crh* cell groups are confined to the secondary prosencephalon. *crh* expressing cell groups in the epiphysis and in the thalamus could be mapped to p2. Based on the revised prosomeric model some of the posterior tubercular *crh* cells could be present in p2 and the rest may be mapped to p3.

*Tyrosine hydroxylase (th)* neuronal cell groups have been well described in the developing and adult zebrafish brain (Holzschuh et al., 2001; Rink and Wullimann 2001). In zebrafish *th* mRNA is expressed in the olfactory bulb, retina, ventral diencephalon, pretectum, locus coeruleus, medulla and in the peripheral nervous system. Two-color in situ hybridization revealed that both *th* and *crh* cells were co-distributed in the posterior tuberculum and in the locus coeruleus. In zebrafish, the *th* positive cells in the locus coeruleus have been identified to be nor-adrenergic (Holzschuh et al., 2001). Several studies using different models have shown that the noradrenergic neurons of the locus coeruleus (LC-NA system) are activated under

stress conditions (Curtis et al., 1997; Morin et al., 1999; Yao et al., 2004). Our results suggest that CRH could possibly function as a neuromodulator or neurotransmitter in the hindbrain LC-NA system.

In the hindbrain, cells positive for *crh* transcripts were detected from 28 hpf and were arranged segmentally. By using rhombomere-specific markers such as *krx-20*, *val* and *hoxb1a*, we mapped the positions of *crh* cells to rhombomere 1 (r1), rhombomere 2 (r2) and rhombomere 4 (r4) at 28 hpf. By 2 dpf, *crh* was also expressed in r3. Co-labeling with *pax6.1* and paraffin sections of 36 hpf and 2 dpf zebrafish brain revealed that *crh* cells were located in the basal plate. Although CRH immunoreactivity and mRNA expression in the hindbrain has been described in other fish species including green molly, goldfish and flounder, this kind of segmental arrangement has not been reported previously (Lu et al., 2004).

In the retina *crh* was expressed in the amacrine cells of the inner nuclear layer which is consistent with the identification of CRH immunoreactivity in the amacrine cells of the retina of goldfish, chicken, turtle and rat (Kiyama et al., 1984; Sakanaka et al., 1987; Yeh and Olschowka 1989).

## **Paper II: Analysis of the expression levels of estrogen receptors in embryonic and adult zebrafish in the absence and presence of exogenous ligands.**

Estrogens in all vertebrate species have an important role in development, growth and reproduction. They mediate cellular signalling by binding to ER. In mammals there are two forms of ERs which are designated as ER $\alpha$  and ER $\beta$  (Jensen 1962; Kuiper et al., 1996). ERs are members of the nuclear receptor superfamily and regulate transcription of genes by binding to E2. Knockout studies in mouse have shown that the two ERs have distinct cellular functions (Couse and Korach 1999; Harris 2007).

Teleost, in contrast to mammals, have three estrogen receptors, one alpha form and two beta forms (Bardet et al., 2002; Pinto et al., 2006). In zebrafish the three *esr* genes are represented as *esr1* (ERalpha), *esr2b* (ERbeta1) and *esr2a* (ERbeta2). In paper II we have analysed the expression levels of the *esr* genes in embryos as well as in adult tissues of zebrafish in the absence and presence of exogenous ligands (E2, DES and 4-NP). Expression levels were measured by quantitative real-time PCR using *beta-actin* as the control gene. Primers specific for the zebrafish *esr* genes were used.

In the absence of exogenous ligands the *esr* transcripts were expressed in all adult zebrafish tissues tested. Consistent with previous studies, *esr1* was highly

expressed in liver (Legler et al., 2000; Meneut et al., 2002; Meneut et al., 2004). Other tissues such as brain, heart and testis also expressed high levels of *esr1* gene. The two beta forms, *esr2b* and *esr2a* were expressed predominantly in liver and also in intestine. High expression levels of *esr2b* were also detected in brain, heart, kidney and ovary. The transcript levels of *esr2a* in intestine and testis were approximately 10% of the levels found in liver. The expression of *esr2a* in brain, eye, swim bladder and kidney represented 5% of the levels measured in liver. In ovary all three *esr* genes showed low level of expression.

Although the embryonic expression patterns of the zebrafish *esr* genes have been described earlier, there is a lack of information on the expression levels beyond early embryonic stages (48 hpf for *esr1* and *esr2b*; 72 hpf for *esr2a*) (Tingaud-Sequeira et al., 2004; Mouriec et al., 2009). Therefore in this paper we analyzed the transcript levels of the three *esr* genes both in embryos and in early larvae. In embryos, all three *esr* genes were detected at a stage before zygotic transcription (<3h) indicating that they are maternally derived. The expression levels of *esr1* gradually increased from 12 hpf onward. During development, *esr2b* transcript levels accumulated from 24 hpf and peaked at the early larval stages (96 hpf and 120 hpf). In contrast to *esr1* and *esr2b*, the expression levels of *esr2a* dropped down to low levels after zygotic transcription (>3h) and began to accumulate at the larval stages (96 hpf and 120 hpf) which is consistent with the results described previously (Tingaud-Sequeira et al., 2004). Variation in the developmental expression profile of the ERbeta subtypes indicate that the two genes have distinct and non-redundant functions during embryonic development.

Auto-regulation of *esr1* in the presence of E2 has been previously described (Carroll and Brown 2006; Meneut et al., 2004). In order to investigate whether the two beta forms could also be regulated by estrogenic ligands, we measured the change in the expression levels of the *esr* genes in embryos, larvae and in individual adult male and female fish following exposure to E2 or synthetic ligands (DES & 4-NP). Based on our dose response experiments for the three ligands, we used 1  $\mu$ M concentration of E2 and 4-NP for adult fish and embryos. In the case of DES we used 100 nM for embryos and 1  $\mu$ M for adult fish.

Exposure of embryos to E2 showed significant upregulation of *esr1* as compared to control groups (0.1% ethanol). Similar results were observed with DES and 4-NP suggesting that *esr1* could be regulated by natural and synthetic ligands. Both E2 and DES were able to activate *esr2b* whereas no effect was detected by 4-NP in

early embryos. The other beta form *esr2a* remained unaffected at embryonic stages after ligand exposure. However, *esr2a* was significantly upregulated by E2 and 4-NP at larval stages. Our results show that the pattern of regulation varies between the two ERbeta subtypes and that they may have diverse function during development.

In order to examine the effects of different ligands on the expression levels of *esr* genes in tissues involved in estrogen signalling, adult male and females were exposed to E2, DES or 4-NP. Exposure of E2 to male or female fish upregulated the expression levels of *esr1* in liver and in testis. Exposure to DES showed increased *esr1* expression in liver of male and female fish and also in brain of female fish. As seen in other fish species we observed significant decrease in *esr1* expression in liver of male fish after exposure to 4-NP (Luo et al., 2005; Sabo-Attwood et al., 2007; Seo et al., 2006). No changes were observed in the expression levels of *esr2b* in tissues of male and female fish following exposure to ligands. However, *esr2a* mRNA levels were significantly reduced in liver of male fish when exposed to E2 or 4-NP. In testis, 4-NP treatment but not E2 or DES induced the expression of *esr2a*.

In mammals, many genes have been identified to be under the control of ERs whereas in teleosts, very few genes have been identified to be regulated by E2 and estrogen mimics e.g. *vtg*, *choriogenin*, *aromatase*, *osteonectin* and *esr1* (Harries et al., 1997; Luo et al., 2005; Lehane et al., 1999; Meneut et al., 2004). Vitellogenin induction is most commonly used as a biomarker in testing the estrogenic potency of xenoestrogens. Our data show that the two ERbeta subtypes, *esr2a* and *esr2b* could be direct or indirect targets of ER ligands. Supporting our results, it was shown in adult male goldfish that ER $\alpha$ , ER $\beta$ 1 and ER $\beta$ 2 are differentially auto-regulated by E2 (Marlatt et al., 2008). Our data also show ligand specific regulation of the *esr* genes and the expression pattern varies between tissues and sex. This study also demonstrates that male fish are more sensitive than female fish and this might be due to the difference in the level of endogenous E2 in both sexes. Finally, our data also show that 4-NP, a synthetic estrogenic compound has both agonist and antagonist properties in tissues of adult fish.

### **Paper III: 4-nonylphenol, a weak estrogenic compound disrupts the neuroendocrine system, notochord and muscle**

4-NP is a microbial breakdown product of nonylphenol ethoxylates that are used in the manufacturing of cleaning products, paints, cosmetics, plastics and herbicides (Naylor et al., 1992; Nimrod and Benson 1996; Tolls et al., 1994). It is a

potent endocrine disruptor capable of mimicking the actions of E2 (White et al., 1994). In paper III we have described the toxicity effects of 4-NP on zebrafish embryos and larvae.

The notochord is an embryonic midline structure that provides axial support to the embryos and is required for the formation of the vertebral column. Signals from the notochord are essential for the patterning of the adjacent tissues such as the neuroectoderm, muscle and heart (reviewed in Stemple 2005). In this study we show that a sub-lethal dose of 4-NP (2  $\mu$ M) was capable of producing distortions or kinks and herniations in the notochord. The embryonic development of 4-NP exposed embryos were compared with their corresponding stage-matched control embryos (0.2% DMSO). All embryos in the control group developed normally. In 4-NP exposed embryos the distortions in the notochord were first visible at 24 hpf and by 2 dpf it became weaker and was only visible as minor kinks at the caudal notochord. By 2 dpf all embryos with distortions or kinks developed herniation(s) at single or multiple sites. Using in situ hybridization we showed that in 4-NP treated embryos, several of the chordamesoderm markers such as *ntl*, *shh*, *ehh* and *col2a1* were persistently expressed in the notochord and in the herniations as late as 50 hpf indicating failure in the differentiation of the notochord. In addition to this, the basement membrane surrounding the notochord was perturbed in embryos exposed to 4-NP. Electron micrographic and histological analyses of 4-NP treated embryos revealed partial loss of the perinotochordal basement membrane at the site of herniation. Embryos with severe notochord undulations displayed gaps in the expression of *col2a1*. We speculate that 4-NP could produce partial and localized damage to the perinotochordal basement membrane either by directly acting on the basement membrane components or by inhibiting the notochord differentiation which in turn disrupts the organization of the perinotochordal basement membrane.

In embryos exposed to 4-NP we observed elevated levels of apoptosis in the notochord, the trunk, the medial fin and the brain. This might be due to cellular damage caused by oxidative stress.

At late larval stages, 4-NP exposed embryos showed varied vertebral defects such as unevenly mineralized vertebral bodies, vertebral curvature and abnormal projections of the haemal arches. It has been reported earlier in chick and zebrafish that ablation of notochord cells during early embryonic stages results in vertebral defects at later stages of development (Christ et al., 2000; Fleming et al., 2004). A recent study has shown that morpholino knockdown of *col27a1a* and

*col27a1b* in zebrafish induced scoliosis (Christiansen et al., 2009). Therefore, we speculate that the disruption of the collagen matrix in the perinotochordal basemembrane of 4-NP exposed embryos could affect the formation of the vertebral column.

Embryos exposed to 4-NP showed reduced motility from 1 dpf and displayed abnormal swimming. Antibody staining revealed mild disorganization of the muscle fibres and aberrant ramifications of the motor neurons. Mechanical analyses of the muscles of both control (DMSO) and 4-NP exposed larvae at the optimal length ( $L_0$ ) showed that in the muscles of some 4-NP exposed larvae, the relaxation from the single twitch stimulation involved an extra contractile phase. Also, the half-time for force increase and for force decay of the single twitch responses were significantly longer in the muscles of the 4-NP treated larvae. In control larvae, tetanic stimulation produced muscular contractions that relaxed to low levels between each stimulation. Whereas in the 4-NP exposed larvae, the muscles showed incomplete relaxation leading to sustained tension level. Impaired relaxation of the muscles in 4-NP treated larvae could explain the abnormal swimming pattern of these larvae. We propose that the poor relaxation and prolonged single twitch contractions could be a result of slow removal of  $Ca^{2+}$  ions from the cell.

In situ hybridization using brain patterning marker genes revealed that there were no obvious changes in the structure of the brain including the neuroendocrine organs, the hypothalamus and the pituitary gland. However, the expression levels of the stress hormone *crh* was significantly elevated in the preoptic area, posterior tuberculum and locus coeruleus. This suggests that transcriptional regulation of CRH could also be mediated through ERs.

Using quantitative real-time PCR we measured the transcript levels of the *lhb* and *fshb*. Our results showed that *fsh* gene expression was undetectable at the stages tested. Interestingly, the mRNA levels of *lhb* was significantly downregulated at 4 dpf in 4-NP treated embryos. These results demonstrate that 4-NP could also disrupt the HPG and HPI axes in zebrafish.

Overall, these results demonstrate that 4-NP affects the embryonic development of zebrafish by disrupting the neuroendocrine system, notochord morphogenesis and muscle function. We speculate that these effects of 4-NP are mediated by different mechanistic pathways. More detailed studies are warranted to unravel the molecular mechanisms involved in these processes.

## 5. CONCLUSIONS

In the first part of this thesis we describe the transcript distribution of *crh* in the developing brain of zebrafish. Localization of *crh* in the hypothalamus is observed as early as 25 hpf. The preoptic nucleus in teleosts projects its axons to the pituitary gland where the hypothalamic neurohormones are released. Hence, detection of *crh* in the preoptic area is suggestive of its hypophysiotropic function. We also detected *crh* in other areas outside the hypothalamus implicating that *crh* may also have non-hypophysiotropic functions. Segmental arrangement of *crh* transcripts was observed in the hindbrain and *crh* transcripts were co-distributed with *th* in the LC-NA system, which is responsible for mediating autonomic and behavioral responses to stress. It is therefore likely that CRH might act as a neuromodulator or a neurotransmitter in the noradrenergic LC. Such an extensive description of *crh* expression during development will be valuable in elucidating the molecular and genetic pathways involved in the specification of *crh* expressing neuroendocrine cell types. This study will also facilitate in dissecting the hypophysiotropic and non-hypophysiotropic role of CRH during zebrafish embryogenesis.

We have shown in paper II that all three *esr* genes are expressed in a wide range of adult tissues of zebrafish, which is consistent with estrogens influence in wide range of physiological processes. Our results on the expression patterns of *esr* genes during development suggests that *esr1* and *esr2b* may be involved in controlling early developmental programming while *esr2a* may be required for later development. Exposure studies with ligands show that *esr1* and *esr2b* are under the regulatory control of ligands during development indicating that ligand-receptor interactions begin already at the time of organogenesis. In adult tissues, only *esr1* and *esr2a* are stimulated in response to ligands in a sex- and tissue specific manner. These results point to us that the ERbeta genes might have distinct roles. Morpholino knockdown studies and ESR mutants may shed some light on the functional significance of the three *esr* genes in zebrafish. Additionally, through this study we also show that 4-NP can have both agonist and antagonist role in adult tissues. This is an important finding which provides a platform to investigate the molecular basis for the dual properties exhibited by 4-NP and understand the effects 4-NP might have on different tissues and cell types.

In paper III we analyzed in detail the developmental toxicity of 4-NP in zebrafish. Our results showed that 4-NP affects the morphogenesis of the notochord by inhibiting notochord differentiation, disrupting perinotochordal basement membrane and affecting the vertebral column formation. Notochord perturbation induces secondary defects in the muscle resulting in impaired relaxation of larval muscles. In addition, 4-NP treatment of embryos perturbs the neuroendocrine axis by altering the transcript levels of *crh* and *lhβ*. The mechanism by which 4-NP targets the notochord is unknown and we speculate that 4-NP might act via diverse pathways. Future efforts should be directed towards dissecting the molecular pathways through which 4-NP targets the notochord. Also, the possibility of ERs influence in the notochord morphogenesis should be investigated.

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