Pituitary-interrenal interactions in zebrafish *(Danio rerio)* interrenal organ development

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Abbreviations and chemical symbols

ACTH	adrenocorticotropin
ACTH-R	ACTH-receptor (MC2R)
bp	base pair
3ß-HSD	3ß-hydroxysteroid dehydrogenase
ß-END	beta-endorphin
BSA	bovine serum albumin
°C	grade Celcius
CaCl2	calcium chloride
CRH	corticotropin-releasing hormone
CY11A1	gene coding for P450 side chain cleavage enzyme (P450scc)
dpf	days post fertilisation
cDNA	complementary DNA
cAMP	cyclic adenosine monophosphate
cds	coding sequence
DAX1	dosage sensitive sex reversal - adrenal hypoplasia congenita gene on the X chromosome, gene 1
DEX	dexamethasone
DßH	dopamine beta hydroxylase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
ENU	N-ethyl-N-nitrosourea
et al.	and others
Fig.	figure
g	gram
GFP	green fluorescent protein
GSP	gene specific primer
GR	glucocorticoid receptor
h	hour
HCl	hydrochloric acid
KH_2PO_4	monopotassium phosphate

HPA axis	hypothalamus-pituitary-adrenal axis
HPI axis	hypothalamus-pituitary-interrenal axis
hpf	hours post fertilisation
dH_2O	distilled water
3ß-HSD	3ß-hydroxysteroid dehydrogenase
ITPG	Isopropyl-ß-D-thiogalactopyranosid
ISH	*In situ* hybridization
kb	kilobase (s); kilobase pairs
LB	Luria Broth
l	liter
M	mole
µ	micro
MC2R	melanocortin 2 receptor (ACTH receptor)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute(s)
m	milli
MSH	melanocyte stimulating hormone
NaCl	sodium cloride
NaOH	sodium hydroxide
$Na_2HPO_4.12H_2O$	disodium hydrogen phosphate dodecahydrate
nt	nucleotide
N-terminus	amino-terminus
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PFA	paraformaldehyde
PCR	polymerase chain reaction
PNMT	phenylethanolamin-N-methyltransferase
POMC	proopiomelanocortin
PTU	phenylthiourea
P450 scc	cytochrome P450 side chain cleavage enzyme
Race-PCR	rapid amplification of cDNA end - PCR
RT-PCR	reverse transcriptase - polymerase chain reaction
RNA	ribonucleic acid

RNase	ribonuclease
rpm	rotations per minute
RT	room temperature
mRNA	messenger RNA
SSC	sodium chloride sodium citrate buffer
StAR	steroidogenic acute regulatory protein
SDS	sodium dodecylsulfate
SF-1	steroidogenic factor-1
Tab.	table
Taq	*Thermophila aquaticus*
U	unit
UV	ultraviolet
V	volt
vs	versus
X-Gal	5-brom-4-chlor-3-indolyl-ß-D-galactopyranosid
wt	wild-type

Gene and protein nomenclature:

The name of genes and proteins from zebrafish, human and mouse are written according to the zebrafish nomenclature guidelines based on *Trends in Genetics* Genetic Nomenclature Guide (1998) (http://zfin.org/zf_info/nomen.html). For example the POMC genes and proteins in zebrafish, human and mouse are written as follows:

species / gene / protein
zebrafish / *pomc* / Pomc
human / *POMC* / POMC
mouse / *Pomc* / POMC

Abstract

In mammals, the pituitary-derived neuropeptide adrenocorticotropic hormone (ACTH) is a major regulator of adrenocortical steroidogenesis and hormone secretion. However, the mechanism by which adrenal growth is governed by pituitary signals and the role of the pituitary in early adrenal development remain to be investigated. In this work the model organism zebrafish was used to elucidate pituitary adrenal interactions during early vertebrate development. The adrenal homologue in zebrafish is located in the head kidney and termed interrenal organ.
This dissertation consists of three parts: (1) cloning and characterization of the zebrafish *pomc* gene, (2) description of interrenal organogenesis in wild-type zebrafish and (3) analysis of pituitary-interrenal interactions by using pituitary mutants, gene-knockdown embryos and pharmacological interventions.

1. The zebrafish proopiomelanocortin (*pomc)* gene

The proopiomelanocortin gene or *pomc* gene in zebrafish shows a similar overall structural organisation as in higher vertebrates including *Homo sapiens*. The gene consists of three exons and two short introns. Intron 1 (339 bp) divides the 5(') untranslated region from the coding region while intron 2 (1522 bp) is located between the signal peptide and the sequence encoding Acth. Transcription start is 26 bp downstream of the TATA box and there is one polyadenylation signal in the 3(') untranslated region. The 964 bp cDNA comprises of an open reading frame encoding a 222 amino acid hormone prepropeptide that is split into six putative hormones. Sequence comparison of zebrafish Pomc to sequences of various other vertebrate species reveals four regions that are highly conserved during the evolution of vertebrates: the N-terminal region, Acth, beta-Msh, and beta-endorphin, whereas the connecting peptides show a higher degree of variability. These findings demonstrate that the zebrafish *pomc* gene closely resembles the gene structure in higher vertebrates, justifying the use of zebrafish as a model organism to study the physiological role of Pomc-derived peptides.

2. Interrenal development and organogenesis in wild-type zebrafish

Development and organogenesis of both steroidogenic and chromaffin components of zebrafish interrenal organ was analysed using whole-mount mRNA *in situ* hybridization (ISH) for a variety of steroidogenic maker genes (*ff1b*, the mammalian *SF-1* (steroidogenic factor 1) equivalence; *cyp11a1* (the gene encoding cytochrome p450 cholesterol side chain cleavage (P450scc) enzyme); *mc2r* (the gene encoding melanocortin 2 or Acth receptor), and *star* (the gene encoding steroidogenic acute regulatory protein) and for the chromaffin marker gene *dßh* (the gene encoding dopamine beta hydroxylase) throughout the developmental stages ranging from 22 hours post fertilisation (hpf) to 7 days post fertilisation (dpf). In addition the enzyme

activity of the interrenal 3ß-hydroxysteroid dehydrogenase (3ß-Hsd) was also examined. The steroidogenic interrenal primordium is earliest detectable at 22 hpf. It appears as bilateral clusters of *ff1b* expressing cells ventral to the 3rd somite. These cell clusters fuse to a single cell mass at 24 hpf, giving rise to expression of key steroidogenic genes *star* and *cyp11a1*. From 4dpf, the steroidogenic interrenal primordium develops further into a distinct bilobed organ lateral to the notochord. The chromaffin interrenal primordium visualised by *dßh* transcripts converges into the steroidogenic interrenal primordium at 2 dpf in form of bilateral cell domains; initially overlapping with the steroidogenic primordium in the right domain. The expanding steroidogenic primordium then also covers the left *dßh* expressing cell domain. After fusion into a single cell mass at 3 dpf, the chromaffin interrenal primordum develops further into a bilobed domain lateral to the notochord consistently in close contact to the steroidogenic cells and eventually appears to be enveloped by the steroidogenic cells. The interrenal development in zebrafish and mammals has a conserved developmental program in respect to both sequential expression of homologous genes and co-development of chromaffin and steroidogenic cells.

3. Pituitary-interrenal interactions during early interrenal development

Zebrafish pituitary-interrenal interactions during development were studied in pituitary mutant embryos (*aal/eya1*and *lia/fgf3* mutants lack different pituitary cell types including corticotrophs; *pit1* mutants have corticotrophs but lack other pituitary cell types), in mc2-receptor (*mc2r*) knockdown morphants and in dexamethasone treated embryos. Interrenal phenotypes of the embryos were assessed by ISH-visualised transcripts of interrenal specific genes of both steroidogenic and chromaffin components of the interrenal organ. Moreover, interrenal phenotypes was analysed at the protein level using histochemical staining for the interrenal 3ß-Hsd. Early interrenal development is fully independent of Pomc/pituitary signalling as demonstrated in *aal/eya1*and *lia/fgf3* mutants lacking pituitary corticotrophs. Until 2 dpf interrenal development assessed by transcripts of steroidogenic genes (c*yp11a1, star, mc2r*) and of the chromaffin gene dopamine ß-hydroxylase (*dßh*) in these mutants remains unaltered as compared to wild-type siblings. However, at 5dpf the pituitary influences expression of the steroidogenic genes at both the transcriptional and the protein level, as evident by the remarkable decrease in expression of all studied steroidogenic genes in these mutants. Expression of *cyp11a1* is decreased to about 50% in *aal/eya1* mutants. Moreover, the size of the interrenal organ is reduced indicating interrenal hypoplasia. Pituitary control of interrenal development resides in pituitary corticotrophs, as *pit1* mutant embryos lacking all pituitary cells except corticotrophs have a similar interrenal phenotype as wild-type embryos. Moreover, there was no difference in interrenal development of *mc2r* knockdown morphants from *aal/eya1* and *lia/fgf3* mutants, indicating that Acth plays a key role in corticotroph action on interrenal development. Inhibition of early steroidogenesis by *mc2r* knockdown induces a

4-fold-upregulation of pituitary *pomc* transcripts in the anterior domain of *pomc* expressing cells only. Accordingly, exogenous dexamethasone suppresses pituitary *pomc* only in the anterior domain, consecutively leading to impaired expression of interrenal steroidogenic genes commencing at 3 dpf. Feedback on the pituitary corticotrophs by glucocorticoids is evident already at 2 dpf and thus, precedes the effects of Pomc derived peptides on the interrenal primordium.

The lack of corticotrophs or the knockdown of *mc2r* not only leads to a decrease in expression of steroidogenic interrenal genes, but also reduces the expression of the chromaffin marker gene *dβh* at later stages of organogenesis. In contrast, 5 dpf dexamethasone-treated embryos which had lost completely anterior pituitary *pomc* expression exhibit normal *dβh* expression. These results indicate a functional interaction between the steroidogenic and the chromaffin cells of the interrenal organ via glucocorticoid secretion, as has also been observed in mammals.

Taken together, these results demonstrate a gradual transition from early pituitary-independent interrenal organogenesis to developmental control by the anterior domain of pituitary corticotrophs acting via mc2 receptors. Interrenal development in the zebrafish shares many conserved molecular and developmental mechanisms with higher vertebrates suggesting that in mammals ACTH is also the key regulator in pituitary-dependent adrenal development. These observations indicate that zebrafish is also a promising model organism for the study of transcription factors involved in human and mouse adrenal development.

Zusammenfassung

Adrenocorticotropin (ACTH) entsteht aus Proopiomelanocortin (POMC) in den corticotrophen Zellen der Hypophyse und ist ein zentraler Regulator der adrenalen Steroidogenese. Allerdings wirkt ACTH *in vitro* antiproliferativ und differenzierend und es ist daher unklar, wie adrenales Wachstum durch die Hypophyse gesteuert wird. Insbesondere liegen widerspüchliche Befunde zum Einfluss der Hypophyse auf die Entwicklung der Nebenniere vor. In dieser Arbeit wurde der Zebrafisch als Modellorganismus eingesetzt, um die Interaktionen zwischen Hypophyse und Nebennieren während der frühen Entwicklung weiter aufzuklären. Das Homolog der Nebenniere beim Zebrafisch wird als Interrenalorgan bezeichnet und ist in die Kopfniere eingebettet.

Die vorliegende Arbeit besteht aus drei Haupteilen: (1) Klonierung und Charakterisierung des Proopiomelanocortin-Gens (*pomc*-Gens) des Zebrafisches, (2) Untersuchung der normalen Entwicklung des Interrenalorgans beim Zebrafisch und (3) Analyse der Wechselwirkung zwischen Hypophyse und Interrenalorgan während der Organogenese des Interrenalorgans durch die Untersuchung verschiedener hypophysärer Mutanten, Gen-knockdown Embryonen und durch pharmakologische Intervention.

1. Das Proopiomelanocortin-Gen (*pomc*-Gen) des Zebrafisches

Das Zebrafisch-*pomc*-Gen besteht aus 3 Exons und 2 Introns und hat eine strukturelle Organisation, die der anderer Wirbeltier-*POMC*-Gene entspricht, einschließlich des humanen *POMC*-Gens. Das Intron 1 (339 bp) liegt zwischen der 5' untranslatierten Region und der kodierenden Sequenz während das Intron 2 (1522 bp) zwischen dem Signalpeptid und der Acth kodierenden Sequenz lokalisiert ist. Die Transkription beginnt 26 bp dowstream der TATA Box und es findet sich ein Polyadenylierungssignal in der 3' untranslatierten Region. Die cDNA ist 964 bp lang und hat eine kodierende Sequenz von 669 bp. Das daraus resultierende Polypeptid mit 222 Aminosäuren dient als Vorstufe für sechs putative Hormone. Vergleicht man die Pomc-Aminosäuresequenz des Zebrafisches mit der anderer Wirbeltiere, so finden sich im Pomc 4 Regionen, die während der Wirbeltierevolution hoch konserviert bleiben: N-terminales Pomc, Acth, ß-Msh und ß-Endorphin. Im Gegensatz dazu ist die Aminosäuresequenz anderer Abschnitte sehr variabel. Diese Daten zeigen, dass das *pomc*-Gen des Zebrafisches der Genstruktur höherer Wirbeltieren ähnelt. Damit erscheint der Zebrafisch geeignet als Modellorganismus zur Untersuchung der physiologischen Rolle Pomc-abgeleiteter Peptide.

2. Die Entwicklung des Interrenalorgans beim Zebrafisch

Die Entwicklung und Organogenese der steroidogenen und der chromaffinen Komponente des Interrenalorgans des Zebrafisches wurden durch *in situ* Hybridisierung (ISH) für die steroidogenen Markergene *ff1b* (Äquivalent des humanen-*SF-1*), *cyp11a1* (kodiert für das Cytochrom p450 Cholesterol Side Chain Cleavage (p450scc) Enzym), *mc2r* (kodiert für Melanocortin 2- bzw. Acth-Rezeptor) und *star* (kodiert für das Steroidogenic Acute Regulatory Protein) sowie für das chromaffine Markergen *dßh* (kodiert für Dopamin-Beta-Hydroxylase) während der Entwicklungsstadien von 22 Stunden nach Fertilisierung (22 hpf, hours post fertilisation) bis zum Tag 7 nach Fertilisierung analysiert. Ergänzend wurde die Enzymaktivität der interrenalen 3ß-Hydroxysteroid-Dehydrogenase erfasst. Das interrenale Primordium ist erstmals 22 hpf als bilateraler Cluster *ff1b*-exprimierender Zellen nachweisbar, die ventral vom 3. Somiten lokalisiert sind. Diese zwei Zellcluster fusionieren dann zu einer gemeinsamen Zellmasse (24 hpf) und führen zur Transkription der steroidogenen Gene *star* und *cyp11a1*. Vom 4. Tag an entwickelt sich der steroidogene Anteil des interrenalen Primordiums weiter zu einem bilobären Organ lateral vom Notochord. Die chromaffine Komponente des interrenalen Primordiums nähert sich der Region des steroidogenen interrenalen Primordiums am Tag 2 als bilaterale Domäne *dßh*-exprimierender Zellen. Diese überlagern sich mit dem steroidogenen Primordium zuerst nur in der rechten Domäne. Im weiteren Verlauf umfasst das steroidogene Primordium dann auch die linke *dßh*-exprimierende Zelldomäne. Nach der Fusion zu einer einzigen Zelldomäne am Tag 3 entwickelt sich das chromaffine Primordium weiter zu einer bilobären Domäne in engem Kontakt mit den steroidogenen Zellen. Die chromaffinen Zellen erscheinen dabei von den steroidogenen Zellen umhüllt. Die Entwicklung des Interrenalorgans beim Zebrafisch zeigt damit, im Vergleich zu Säugetieren, ein konserviertes Entwicklungsprogramm hinsichtlich der sequenziellen Expression homologer Gene und der Co-Entwicklung von chromaffinen und steroidogenen Zellen.

3. Der Einfluss der Hypophyse auf die Entwicklung des Interrenalorgans

Die Interaktion zwischen Hypophyse und Interrenalorgan während der Entwicklung wurde mit Hilfe der hypophysären Mutanten *aal/eya1, lia/fgf3* und *pit1,* durch *mc2r*-Rezeptor (*mc2r*)-knockdown Embryonen und Dexamethason-behandelte Embryonen analysiert. Bei der *aal/eya1* und der *lia/fgf3* Mutante fehlen unterschiedliche hypophysäre Zellpopulationen, einschließlich der corticotrophen Zellen; die *pit1* Mutante besitzt corticotrophe Zellen während die anderen hypophysären Zellpopulationen fehlen. Der interrenale Phänotyp der jeweiligen Embryonen wurde mittels mRNA-ISH für spezifischen Gene der steroidgenen und chromaffinen Komponente des Interrenalorgans analysiert. Ergänzend wurde die Enzymaktivität der interrenalen 3ß-Hydroxysteroid-Dehydrogenase erfasst. Die frühe interrenale Entwicklung ist vollständig unabhängig von der Hypophyse, wie durch die *aal/eya1*

und *lia/fgf3* Mutanten belegt wird. Bis zum Tag 2 nach der Fertilisierung bleibt bei diesen Mutanten die interrenale Entwicklung unverändert im Vergleich zu Wildtyp-Embryonen, was durch Transkripte steroidogener Gene (*cyp11a1, star, mc2r*) und chromaffiner Gene (*dßh)* analysiert wurde. Am Tag 5 beeinflusst die Hypophyse transkriptionell und translational die Expression aller steroidogenen Gene. Im Vergleich zu den Wildtyp-Embryonen, ist die Transkription von *cyp11a1* bei der *aal/eya1* Mutante um 50% verringert. Außerdem ist die Größe des Interrenalorgans bei dieser Mutante reduziert als Hinweis auf die Entwicklung einer interrenalen Hypoplasie. Die hypophysäre Steuerung der interrenalen Entwicklung erfolgt ausschließlich durch die corticotrophen Zellen der Hypophyse, da die *pit1* Mutante einen normalen interrenalen Phänotyp besitzt. Da der interrenale Phänotyp der *mc2r*-knockdown Morphanten sich nicht von dem der *aal/eya1* und *lia/fgf3* Mutanten unterscheidet, besitzt Acth offenbar die Schlüsselrolle in der Vermittlung der Wirkung der corticotrophen Zellen auf die interrenale Entwicklung. Die Hemmung der frühen Steroidogenese durch den Knockdown des *mc2r* verursacht eine Hochregulation der *pomc*-Expression ausschließlich in der anterioren Domäne der *pomc*-exprimierenden Zellen der Hypophyse. Entsprechend supprimiert exogenes Dexamethason auch nur die anteriore hypophysäre *pomc*-Expression. Die durch Dexamethason supprimierte *pomc*-Expression führt zu einer verringerten Expression des steroidogenen Gens *cyp11a1* ab Tag 3. Ein Feedback durch Glukokorticoide auf hypophysärer Ebene ist dagegen bereits am Tag 2 nachweisbar. Damit geht er der Wirkung Pomc-abgeleiteter Peptide auf das interrenale Primordium voraus. Der Mangel an corticotrophen Zellen oder ein Knockdown des *mc2r* führt nicht nur zu einer Abnahme der Expression der steroidogenen interrenalen Gene, sondern auch zu einer verringerten Expression des chromaffinen Markergens *dßh* in den späteren Stadien der Organogenese. Eine normale *dßh*-Expression wird dagegen in den Dexamethason-behandelten Embryonen am Tag 5 beobachtet, obwohl die anteriore hypophysäre *pomc*- Expression und die interrenale Steroidogenese supprimiert ist. Diese Daten belegen, dass die funktionelle Interaktion zwischen steroidogenen und chromaffinen Zellen des Interrenalorgans entscheidend durch Glukokorticoide vermittelt ist.

Zusammenfassend zeigen die Ergebnisse einen schrittweisen Übergang von einer frühen hypophysenunabhängigen Phase der interrenalen Organogenese zu einer Entwicklungssteuerung durch die anteriore Domäne der corticotrophen Zellen der Hypophyse, deren Wirkungen entscheidend über den Mc2-Rezeptor vermittelt werden. Die Entwicklung des Interrenalorgans beim Zebrafisch zeigt im Vergleich zu Säugetieren in vieler Hinsicht hochkonservierte Entwicklungsprozesse. Der Zebrafisch ist damit ein vorzüglich geeigneter Modellorganismus, um zukünftig auch die Rolle von Transkriptionsfaktoren, die an der Nebennierenentwicklung bei Mensch und Maus beteiligt sind, weiter zu klären und um neue Entwicklungsgene (z.B. im Rahmen von Mutagenese-Screens) zu identifizieren.

1 Introduction

1.1 The hypothamamus-pituitary-adrenal axis (HPA axis)

1.1.1 HPA axis and glucocorticoid feedback regulation

The hypothalamic-pituitary-adrenal axis (HPA axis) is the neuroendocrine system regulating essential reactions of stress responses. Its functions are mediated via controlling steroidogenesis in the adrenal cortex.

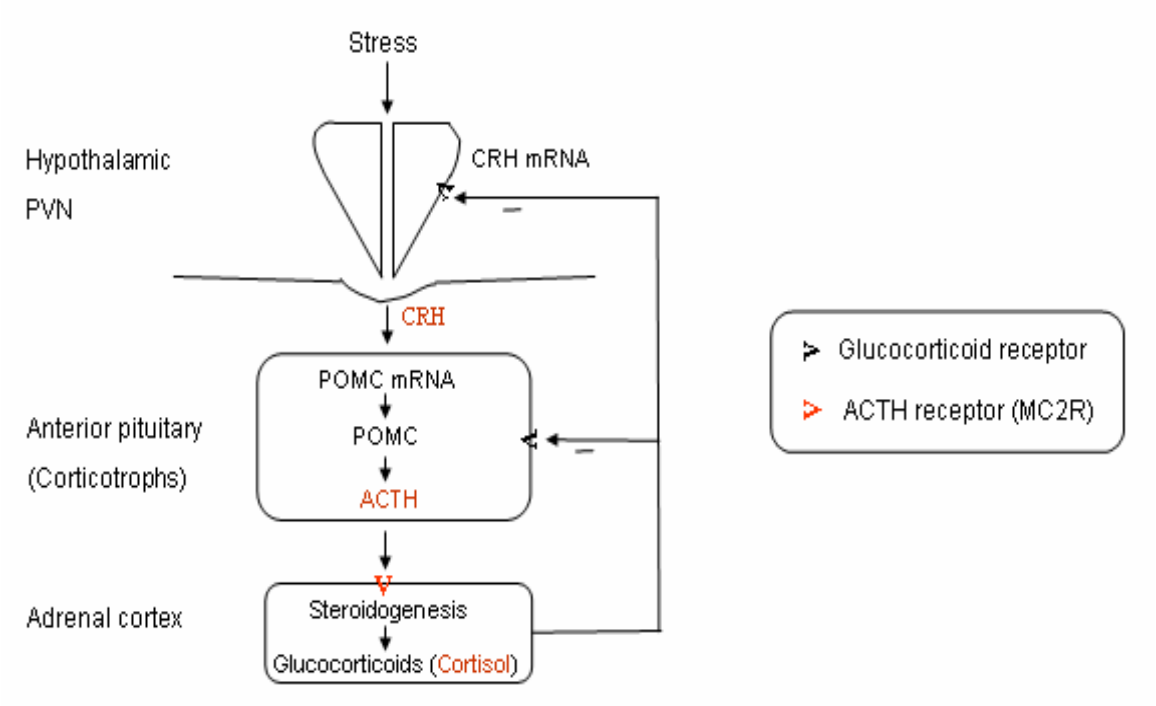

Fig. 1. The hypothalamo-pituitary-adrenal (HPA) axis and glucocorticoid feedback regulation. Parvocellular neurons in the paraventricular nucleus (PVN) produce corticotropin-releasing hormone (CRH) which in turn stimulates adrenocorticotropin (ACTH) synthesis and release from the anterior pituitary corticotroph cells. ACTH then initiates steroidogenesis via ACTH receptor and stimulates glucocorticoid (cortisol) release from the adrenal cortex. Glucocorticoids act at multiple loci within the body to maintain homeostasis. Due to the damaging effects of extended glucocorticoid exposure the HPA axis is tightly regulated. Glucocorticoids feedback, via glucocorticoid receptors in the PVN and the anterior pituitary, to decrease HPA activity. POMC, proopiomelanocortin; PVN, paraventricular nucleus. (taken and modified from Matthews, 2002).

In response to stress, corticotropin-releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus. CRH then stimulates corticotrophs in the anterior lobe of the pituitary gland to produce POMC peptide and POMC is further

processed to adrenocorticotrophic hormone (ACTH) to be secreted into the circulation. ACTH targets ACTH receptors (melanocortin 2 receptors (MC2R)) on the surface of adrenocortical cells and triggers a series of signal transductions, resulting in increased adrenal steroidogenesis. The produced cortical steroid hormones, mainly cortisol, then take part in regulating multiple pathways including carbohydrate metabolism to gain energy for the organism to cope with the stress. As soon as the balance is regained, the excessive output of steroid products, mainly cortisol, will feedback at pituitary and hypothalamus levels to inhibit further secretion of ACTH in the anterior pituitary as well as CRH in the hypothalamus (Fig. 1).

1.1.2 Ontogeny of the HPA function

1.1.2.1 Higher vertebrates

In birds and mammals, the HPA is significantly activated during late fetal and early neonatal life, followed by a period after birth called stress-hyporesponsive period in which stressors fail to elicit an increase in circulating levels of glucocorticoids (for review see Kapoor et al., 2006). The timing of maturation of the HPA axis is highly species specific and is related to brain development (Dobbing and Sands, 1979). In sheep and guinea pigs, animals that give birth to mature offspring, brain growth and neuroendocrine maturation occur largely *in utero*, whereas in animals giving birth to immature newborns (e.g. rats, rabbits, mice) neuroendocrine development takes place mainly in the postnatal stages. In humans, horses, pigs, sheep and guinea pigs, rapid maturation of the HPA function is observed during fetal life, as their fetal plasma cortisol levels increase exponentially in the last 10 days of gestation preceded by an increase in fetal ACTH (for review see Kapoor et al., 2006). At first glance it seems paradoxical that high cortisol levels parallel high circulating ACTH for such a long time, as glucocorticoids are known to negatively feedback on HPA axis function (Matthews and Challis, 1995; McCabe et al., 2001; Unno et al., 1998). Further analysis has revealed increased central drive at the level of the fetal paraventricular nucleus (CRH and arginine vasopressin) in late gestation that may alter the glucocorticoid feedback sensivity at various levels within the HPA axis (for review see Kapoor et al., 2006). This mechanism may serve the high glucocorticoid requirements of the fetus during this developmetal stage, as glucocorticoids are known to be essential for

the maturation of many organ systems such as lung, liver, kidney as well as for brain and neuroendocrine development (Liggins, 2000). Negative feedback regulation of glucocorticoids at different levels of the HPA axis was described in fetal life in sheep, guinea pigs and mice (Matthews and Challis, 1995; McCabe et al., 2001; Reichardt and Schutz, 1996; Unno et al., 1998). This negative feedback was shown to be mediated via glucocorticoid receptors (GR), as in wild-type mice it is established around day E 16.5 of embryonic development whereas in glucocorticoid receptor deficient mice it is not found (Reichardt and Schutz, 1996).

1.1.2.2 Teleosts

As the adrenal organ equivalent in fish is called interrenal organ, the HPA axis homologue in fish is abbreviated as HPI axis. In many fish, cortisol is found in both unfertilized and newly fertilized eggs but rapidly decreases in the earliest stages of embryogenesis until hatching when the HPI axis has been shown to be functionally established (Barry et al., 1995a; Hwang et al., 1992; Stouthart et al., 1998). The availability of cortisol in eggs suggests maternal deposition of this hormone in eggs during oogenesis and demand of embryos for glucocorticoids during early embryogenesis (Barry et al., 1995a; Hwang et al., 1992; Stouthart et al., 1998). Ontogeny of interrenal steroid biosynthesis has been reported in many fish mostly after hatching: Japanese flounder *(paralichthys olivaceus)* after 2 weeks, tilapia (*Oreochromis mossambicus*) after 1 day (Hwang and Wu, 1993; Hwang et al., 1992), rainbow trout (*Oncorhynchus mykiss)* after 1 day (Barry et al., 1995a; Hwang et al., 1992), milkfish *(Chanos chanos)* after 3 days (Hwang et al., 1992). In contrast, in common carp (*Cyprinus carpio)*, endogenous cortisol was observed before hatching (hatching at 56-72 hpf) at 36 hpf and was preceded by endogenous ACTH and alpha-MSH secretion (Stouthart et al., 1998). However, the capability of the interrenal tissue to produce steroids does not necessarily mean that the HPI axis as a whole is integrated and capable of responding to stress with an increase in cortisol production. Barry et al. showed that interrenal tissue of rain bow trout produced significant cortisol in response to 1-24 ACTH before and after hatching *in vitro* (Barry et al., 1995b) and endogenous cortisol was observed already by week 1 after hatching *in vivo* (Barry et al., 1995a). However, only until 2 weeks after hatching the HPI axis was found to be functionally integrated and responsive to stress *in vivo* (Barry et al., 1995a). In common carp, whole body cortisol

levels are increased by stressors (mechanical pressure) only from 50 hpf onwards indicating that the HPI axis in carp is fully functional at the time of hatching. Thus, it is suggested that the functional integration of the HPI axis occurs concomitantly with the maturation of the hypothalamic or sensory components of the system, rather than at the level of interrenal cells, as has also been suggested by studies in mammals (Stouthart et al., 1998).

Little is known about the ontogeny of negative feedback of glucocorticoids at different levels within the HPI axis in fish. Barry et al. 1995 suggested that negative feedback mechanisms within the HPI axis develop only 3 to 4 weeks after hatching in rainbow trout (Barry et al., 1995a).

1.2 The adrenal structure in mammals and teleosts

1.2.1 Adrenal structure in mammals

Mammalian adrenal glands are endocrine glands located on top of both kidneys. Each gland is composed of two parts that are different in histological structure, function and embryonic origin: an outer part (adrenal cortex) that secretes steroid hormones and an inner part (adrenal medulla) that releases catecholamines (adrenaline and noradrenaline). Adrenal hormones control many vital functions of the body related to stress responses such as energy, electrolyte metabolism, inflammatory response, heart rate, blood pressure.

The adrenal cortex is derived from mesoderm (Else and Hammer, 2005). As a steroidogenic tissue, its cells are characterized by numerous lipid droplets, preponderance of smooth endoplasmic reticulum and tubulovesicular mitochondria in their cytoplasm. The adrenal cortex is layered into three concentric zones of steroid-synthesizing cells, from outside to inner part: zona glomerulosa, zona fasciculata, zona reticularis, differing in their characteristic arrangement of cells and major steroid hormones (Fig. 2**)**. Mineralocorticoids (mainly aldosterone) are secreted from the outermost layer zona glomerulosa in response to increased potassium levels or decreased blood flow to the kidneys as a part of the renin-angiotensin system. Aldosterone controls the concentration of electrolytes, mainly by increasing sodium and water reabsorption as well as potassium excretion. Glucocorticoids

(cortisol in humans and corticosterone in mice) and weak androgens (e.g., dehydroepiandrosterone (DHEA)) are produced by zona fasciculata and zona reticularis, respectively, under the stimulation of adrenocorticotropic hormone (ACTH, corticotropin). Cortisol is involved in regulating carbohydrate metabolism and immune functions. Its actions are mediated by intracellular glucocorticoid receptors via altering target gene expression (Morsink et al., 2006; Payne and Adcock, 2001; Schoneveld et al., 2004; Yudt and Cidlowski, 2002).

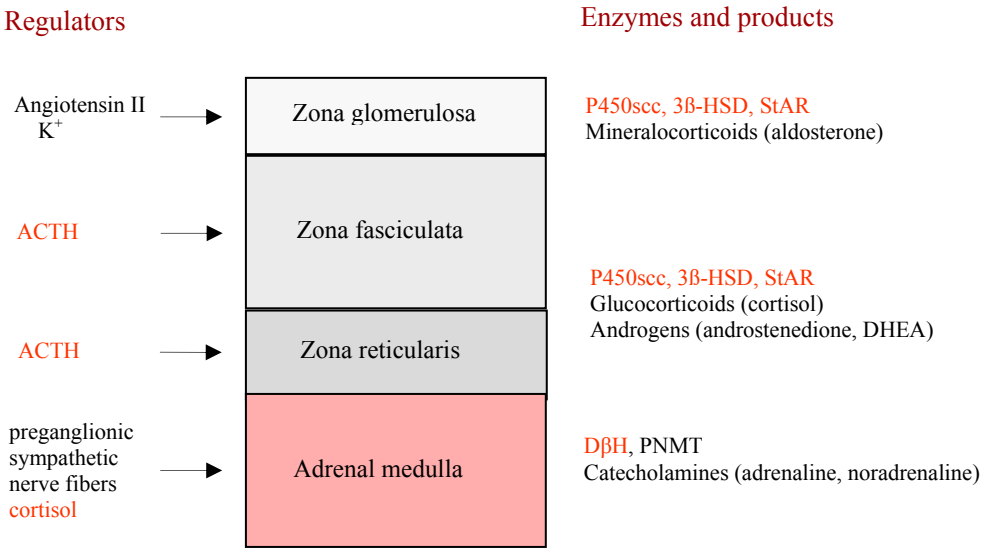

Fig. 2 Schematic structure of the adrenal gland in man. Regulators , enzymes and products of steroidogenesis of each layer of the gland (taken and modified from Kacsoh, 2000).

Adrenal medulla is derived from the embryonic neural crest (Unsicker et al., 2005). It is composed mainly of catecholamine producing chromaffin cells and is the site of the conversion of the amino acid tyrosine into the catecholamines epinephrine and norepinephrine (also called adrenaline and noradrenaline, respectively) by a number of specific enzymes like tyrosin hydroxylase (TH), dopamin ß hydroxylase (DßH), phenylethanolamin-N-methyltransferase (PNMT). Medullary catecholamine secretion is under sympathetic control and paracrine control of adrenal cortex products (e.g. cortisol) (Kacsoh, 2000).

1.2.2 Interrenal organ of teleosts

The adrenal gland in fish is called the interrenal organ, as it is embedded in the head kidney. It consists of two kinds of secretory cells with varying morphology in different teleost species. Besides steroidogenic interrenal cells forming the interrenal component homologous to the adrenal cortex, there are also chromaffin cells forming the interrenal component equivalent to the adrenal medulla in higher vertebrates (Grassi Milano et al., 1997; Nandi, 1962; Rocha et al., 2001). In contrast to the structural organisation in the mammalian adrenal gland that consists of distinct adrenal cortex and medulla, the teleost interrenal organ has been described as composed of highly intermingled steroidogenic and chromaffin cells. Nandi J. et al. 1962 have described the structural organisation and the distribution of steroidogenic and chromaffin cells of the interrenal organ in 129 teleost species (Nandi, 1962). The interrenal organ consists of streaks, lumps or cell strands, which are located around the post cardinal veins or their branches (Nandi, 1962). The distribution of steroidogenic and chromaffin cell components in relation to the post cardinal vein is divided in 4-5 subtypes. The family of the Cyprinidae, to which the zebrafish belongs, exhibits steroidogenic component of the type II and a chromaffin component of the type IV. Here the steroidogenic interrenal cells are mostly located around the small or medium branches of the post cardinal veins, and therefore distribute far in the head kidney, while they are mixed with the chromaffin interrenal cells (Nandi, 1962). The interrenal organ of many teleosts is asymmetrically located and frequently found on one side only (Grassi Milano et al., 1997; Nandi, 1962; Rocha et al., 2001).

1.3 Adrenal steroidogenesis

1.3.1 In mammals

The precursor of all steroid hormones is cholesterol. The pathways of steroid biosynthesis in the adrenal cortex are shown in Fig. 3. Steroid hormone production is performed by cholesterol-processing enzymes. The two main categories of these enzymes are cytochrome P450 (CYP) enzymes and hydroxysteroid dehydrogenases. In a first step cholesterol is mobilized from cholesterol esters stored in lipid droplets in the cytoplasm

and transported by steroidogenic acute regulatory (StAR) protein to the inner mitochrondrial membrane. Here cholesterol is converted to pregnenolone by side chain cleavage enzyme (P450scc). This is the first and rate-limiting step in the synthesis of all steroids, hence this step is a key regulatory step controlled by a number of physiological regulators. Pregnenolone itself is not a hormone, but it is the immediate precursor for the synthesis of all steroid hormones. From here, the steroidogenesis branches out to 3 different pathways leading to formation of 3 major classes of steroid hormones: mineralocorticoids (aldosterone), glucocorticoids (cortisol), and androgens (DHEA, androstendione) (Hsu et al., 2006a; Keegan and Hammer, 2002) (Fig. 3).

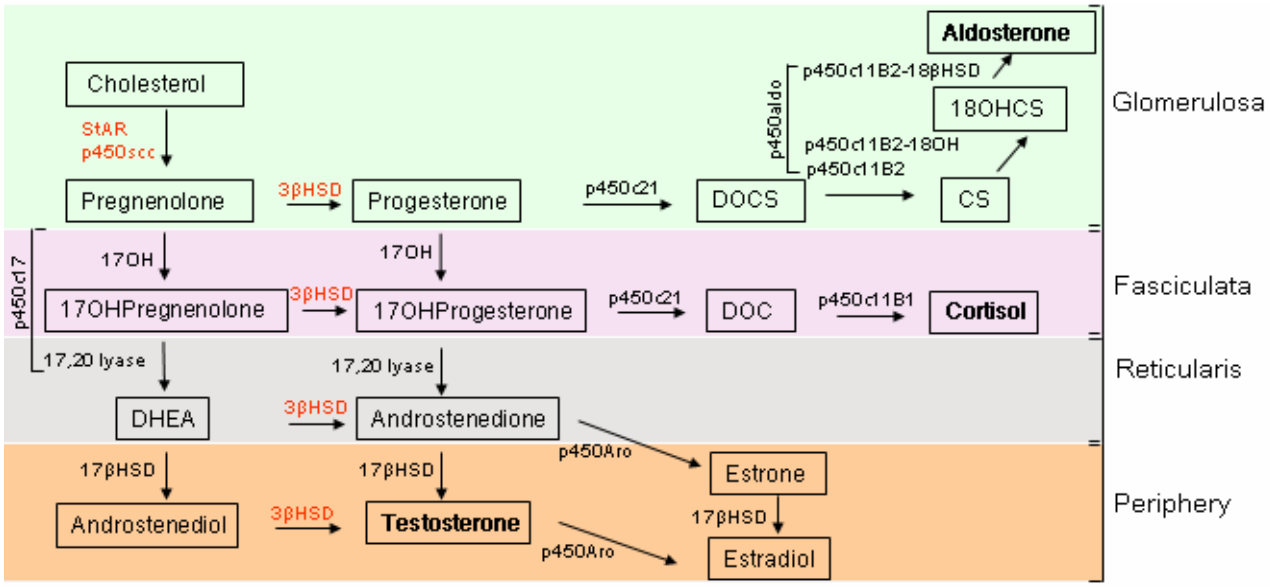

Fig. 3 Steroidogenesis in the human adult adrenal cortex. The biosynthetic pathways for mineralocorticoid, glucocorticoid and adrenal androgen production from the common precursor cholesterol are outlined for the three zones of the adrenal cortex (outer glomerulosa, middle fasciculata and inner reticularis). Zone- and cell-specific steroid production is accomplished by the restricted synthesis of (1) the specific peptide hormone receptors and (2) a specific set of steroidogenic enzymes unique to a given steroid hormone biosynthetic pathway. Abbreviations: CS, corticosterone; DHEA, dehydroepiandrosterone; DOC deoxycortisol; DOCS deoxycorticosterone; 18OHCS, 18-hydroxylase corticosterone; p450scc, side-chain cleavage enzyme; p450c17, enzyme complex containing 17-hydroxylase (17OH) and 17,20 lyase activities; p450c21, 21-hydroxylase; p450Aro, aromatase; p450aldo, aldosterone synthase, the enzyme complex containing 11β-hydroxylase (p450c11B2), 18-hydroxylase (18OH) and 18β-hydroxysteroid dehydrogenase (18βHSD) activities; p450c11B1, 11β1-hydroxylase; 3βHSD, 3β-hydroxysteroid dehydrogenase; 17βHSD, 17β-hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein *(taken and modified from Keegan and Hammer, 2002).*

1.3.2 In teleosts

In fish, cortisol is the principal product of interrenal steroidogenesis. In adult fish, the plasma concentration of cortisol rises dramatically during stress (Mommsen et al., 1999). The absence of a unique mineralocorticoid and the unusual structure of the fish glucocorticoid receptor suggest that cortisol serves both glucoccorticoid and mineralocorticoid functions (for review see Mommsen et al., 1999). In teleosts, cortisol is not only an essential component of the stress response but also a significant regulator for osmoregulation, growth and reproduction (Kahri et al., 1996; Miguel Mancera et al., 2002; Varsamos et al., 2005; Yada et al., 2005).

The biosynthesis of cortisol in fish is similar to that in mammals and involves a microsomal enzymatic pathway, including P450c21, P450c17 and 3beta-hydroxy steroid dehydrogenase (3ß-Hsd). Moreover, fish possess mitochrondrial inner membrane monooxygenase p450scc and the 11ß-hydroxylase catalysing the 11ß-hydroxylation of deoxycortisol/deoxycorticosterone (Mommsen et al., 1999; Sandor et al., 1984). The secretion of cortisol is controlled by the hypothalamus-pituitary-interrenal axis (HPI axis). ACTH released from the pituitary is the main stimulator of cortisol secretion from the interrenal organ. Several factors, including hormones, stress and negative feedback of cortisol at the level of the hypothalamus and the pituitary can modulate ACTH secretion in fish, and therefore cortisol production (Mommsen et al., 1999).

1.4 Development of the mammalian adrenal gland and its regulators

Adrenal development contains sequential evens of cell determination, migration and coalescence of the two discrete structures that are derived from distinct embryonic origins.

Regulators as well as mutants and their homologues defined at each developmetal stage of adrenal cortex were recently elegantly reviewed by Else and Hammer, 2005.

The origin of the adrenal cortex is still not fully clear. It is believed to derive from coelomic epithelium of the urogenital ridge and/or the underlying mesenchyma that also gives rise to the gonads and the kidneys (Else and Hammer, 2005; Mesiano and Jaffe,

1997). Adrenocortical development and growth can be divided into three consecutive stages as outlined in Fig. 4 (Else and Hammer, 2005): the initial specification of the adrenogonadal and subsequent adrenal primordium, the functional differentiation and zonation of the gland, the growth and organ maintenance of the adult gland. Each stage is distinct by its molecular requirements. Early stages are regulated by a program of sequential gene expression, influenced by local paracrine signalling molecules and mainly independent of endocrine regulators of adult adrenal function and later stages (Fig. 4, a and b) (Else and Hammer, 2005). Later stages are mainly governed by the endocrine regulators that are peptides derived from pituitary proopiomelanocortin (POMC) (Fig. 4,c and d). Among identified genes regulating early stages of adrenal development, the gene encoding the orphan nuclear receptor sterodogenic factor 1 (*SF-1*, also termed *NR5A1*) has been found to be essential and specific for development of the adrenogonadal primordium. It is the earliest marker differentiating the adrenogonadal primordium from the urogenital ridge and is a transcription factor regulating expression of various steroidogenic genes including the rate limiting gene encoding cholesterol side chain cleavage enzyme (scc) (Lala et al., 1992; Rice et al., 1991; Val et al., 2003). Mice deficient in SF-1 die perinatally and demonstrate complete absence of both the adrenal gland and the gonads (Ozisik et al., 2003). Moreover, SF-1 has been shown to have functional interactions with DAX1 (*NR0B1*), another nuclear receptor specifically regulating adrenogonadal development (Lalli and Sassone-Corsi, 2003). SF-1 directly activates the *Dax1* promotor, wheares DAX1 antagonizes SF-1 mediated transcriptional activation *in vitro* (Lalli and Sassone-Corsi, 2003).

The adrenal medulla develops from ectodermal neural crest cells known as sympathoadrenal (SA) cell lineage which also gives rise to sympathetic neurons. Among a variety of identified transcription factors involved in the generation of SA from neural crest cell, the helix-loop-helix transcription factor MASH-1 and the homeodomain transcription factor PHOX2B are known to play key roles in the early differentiation of SA cells. Mice deficient in MASH-1 show arrested chromaffin cells at the neuroblast stage and lack of catecholaminergic differentiation (Hueber, 2002). Also, *Phox2b*-/- mice exhibited arrested chromaffin cells at an even earlier stage of development, lack of the catecholaminergic marker TH and fail to form a centrally located medulla (Hueber, 2005).

Moreover, glucocorticoids secreted from the adrenal cortex are often believed to act as local factors regulating the determination of medullary chromaffin cells fate (for review see Unsicker et al., 2005). However, this theory has been criticized by some recent *in vivo* studies in *Gr*-/- and *Sf1*-/- mice (for details see 1.5).

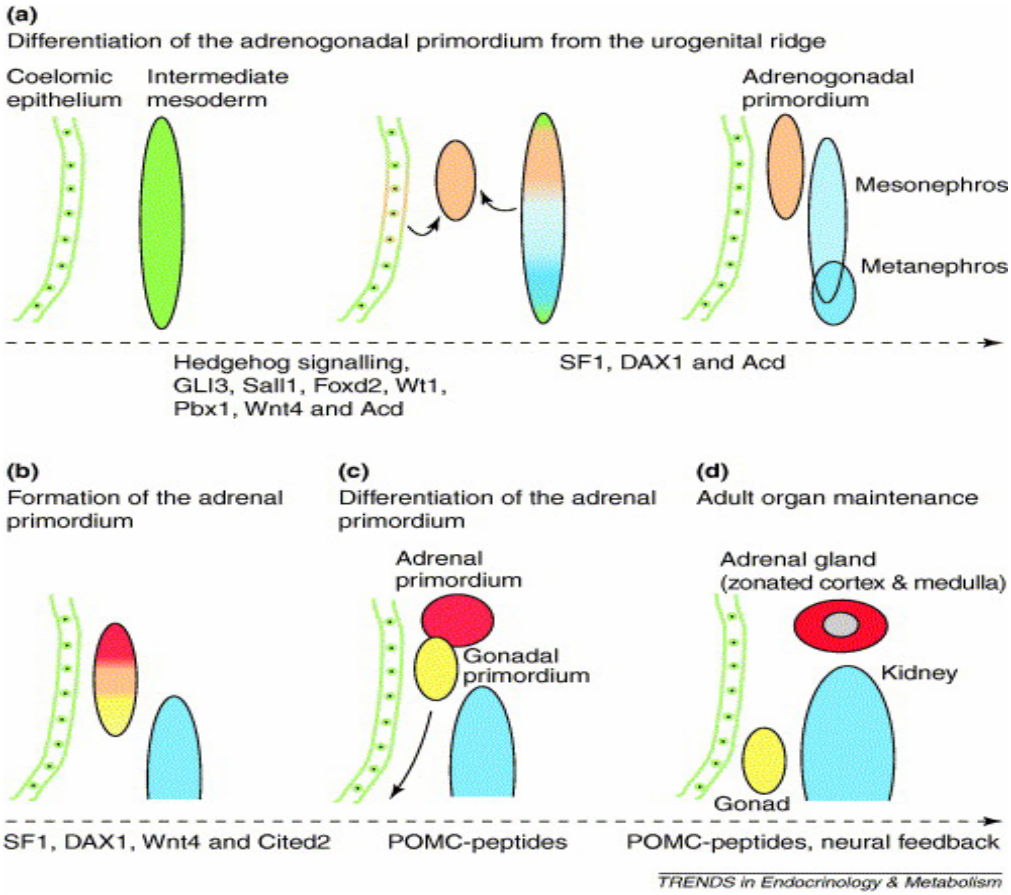

Fig. 4. Adrenocortical development: from the urogenital ridge to the adult adrenal gland. Within the urogenital ridge, the adrenogonadal primordium is formed by cells from the coelomic epithelium and/or intermediate mesoderm (a). Cells of the intermediate mesoderm also give rise to renal precursors (mesonephros) and the definitive kidney (metanephros). Within the adrenogonadal primordium, the adrenal primordium becomes evident (b) and further differentiates into adrenocortical steroidogenic cells (c). The adult adrenal gland is localized cranial to the kidney (d). In parallel to the depiction of adrenocortical development, factors are annotated which have been implicated in these steps, either by human genetic diseases (capital letters) or mouse models (first letter capitalized). Abbreviations: Sall1, Sal-like 1; Wt1, Wilms tumor 1 (taken from Else and Hammer, 2005)

In mouse, the adrenogonadal primordium is formed at embryonic day 9 (E9) as detected by expression of *Sf1* and *Dax1* (Keegan and Hammer, 2002; Val et al., 2003). Separation of

adrenal primordium from gonadal primordimum takes place from E9.5 to E13 (Val et al., 2003). Earliest expression of steroidogenic enzymes is detectable in the adrenal primordium at E11 (Keegan and Hammer, 2002; Val et al., 2003). Migration of neural crest derived primodium to the adrenal primordium occurs from E12 to E14, followed by the zonation of adrenal cortex and movement of medullary cells to the centre of the organ. The adrenal cortex is encapsulated from E14.5 to E16.5. At this stage distinct cortical and medullary zones have been formed, with the cortex surrounding the medulla (Val et al., 2003).

1.5 Intra-adrenal interactions in vertebrates

Adrenal medulla and cortex are interdispered to various degrees in mammals and also in non mammalian vertebrates. The adrenal cortex and medulla are in close spatial association, even in mammals whose adrenal medulla and cortex are considered to be distinct parts. The occurrence of chromaffin cells in the adrenal cortex and vice versa suggest functional interactions between these cell types (for review see Ehrhart-Bornstein et al., 1998; Unsicker et al., 2005).

Data have shown evidence for a direct influence of medullary products on cortical function. Epinephrine or norepinerphrine increase corticosteroid secretion from the isolated porcrine adrenal gland when perfused with these hormones (Bornstein et al., 1990; Ehrhart-Bornstein et al., 1994). This effect of catecholamines was found to be mediated via increased transcription of genes coding for steroidogenic enzymes in adrenocortical cells (Ehrhart-Bornstein et al., 1991; Guse-Behling et al., 1992). Furthermore, chromaffin cells per se appear to have a direct role in the regulation and mainternance of cortical function, as cortisol released from cortical cells in coculture with chromaffin cells was 10-fold increased compared to that from the same number of isolated pure cortical cells, although catecholamines released from the chromaffin cells of the coculture contribute only 20% to this increase (Haidan et al., 1998).

Conversely, the effects of adrenal cortex products on medullary functions and differentiation have been well characterized. Glucocorticoids have long been known to be essential for expression of phenylethanolamine N-methyl transferase (PNMT), an adrenaline-forming enzyme of the chromaffin cells, as DEX can restore this enzyme

activity that was dramatically impaired in hypophysectomized rats (Wurtman and Axelrod, 1966). Further analysis showed that this effect of DEX is via increasing the rate of synthesis of the enzyme protein but not directly via the enzyme activity itself (Wurtman and Axelrod, 1966). The effect appeared specific, as DEX was not capable of stimulating other catecholaminergic enzymes like tyrosine hydroxylase (Wurtman and Axelrod, 1966). Acordingly, reduced PNMT expression was observed in 21-hydroxylase deficient mice harbouring a defect that causes glucocorticoid and mineralocorticoid difficiency (in man it is the most common genetic defect causing congenital adrenal hyperplasia (CAH)). The reduced PNMT expression in these mutant mice goes along with low adrenal catecholamine levels indicating severe adrenomedullary dysfunction (Bornstein et al., 1999).

With respect to chromaffin cell development, it has been assumed that glucocorticoid signalling is essential for chromaffin cell determination and development. Data gained *in vitro* in the last two decades have supported this theory. Several investigations have demonstrated that glucocorticoids are needed for two important sequential steps in chromaffin cell development: first to suppress neuronal markers in sympathoadrenal (SA) progenitor cells turning them towards a chromaffin cell phenotype and second, to induce the adrenaline synthesizing enzyme PNMT (Anderson and Axel, 1986; Bohn et al., 1981; Michelsohn and Anderson, 1992). Moreover, glucocorticoids can inhibit the shift of chromaffin cell phenotype to neuronal phenotype of young chromaffin cells stimulated by nerve growth factor (NGF) (Doupe et al., 1985; Seidl and Unsicker, 1989; Unsicker et al., 1978)**.** However, *in vivo* data obtained from glucocorticoid receptor-null mice (*Gr*-/-) of Unsicker's group (Finotto et al., 1999) strongly contradict the doctrine of an essential role of glucocorticoid signalling for the development of chromaffin cells. The deficiency of GR in this mouse model only affected expression of PNMT, as also observed in hypophysectomized rats (Wurtman and Axelrod, 1966) and *Crh* null mice (Yoshida-Hiroi et al., 2002). Otherwise, *Gr*-/- mice have an unaltered number of chromaffin cells that have a phenotype indistinguishable to wild-type mice with all structural and chemical aspects. The data also clearly showed that chromaffin cells are not converted to a neuronal phenotype in absence of GR-signalling as one would expect from the "glucocorticoid theory". Accordingly, the most recent study from Unsicker group's in *Sf-1* null mice having no adrenal cortex also demonstrated that the adrenal cortex (including

glucocorticoids) is not an prerequisite for chromaffin cell determination and differentiation, as in *Sf-1*-/- mice 50% of the chromaffin cells with all normal features including apoptotic and proliferative cell rates, except lack of PNMT expression were found in the "adrenal anlage" at E 13.5 and E 17.5 compared to that of wild-type mice. However, the loss or missing 50% fraction of chromaffin cells suggests that the adrenal cortex is necessary for proper migration of SA progenitors to the adrenal anlage (Gut et al., 2005).

Little is known about the interactions between the steroidogenic and chromaffin components in the interrenal organ in fish. However, cortisol has been shown to increase catecholamine release from chromaffin stores in the rainbow trout (Reid et al., 1996). Vice versa, catecholamine is reported to stimulate cortisol release in sea bass *(Dicentrachus labrax)* (Rotllant et al., 2006). The availability of the zebrafish model with appropriate mutants and the gene knockdown technique provides an excellent tool to further elucidate this issue.

1.6 Proopiomelanocortin (POMC) and adrenal function and development

1.6.1 POMC and POMC derived peptides

Proopiomelanocortin (POMC) encoded by the *POMC* gene is a polypeptide precursor of several peptide hormones that play important roles not only in the regulation of the hypothalamus-pituitary-adrenal axis, adrenal development and function, but also in feeding, pigmentation and other vital physiological processes. The *POMC* gene is expressed in various tissues, mainly in pituitary and hypothalamus, but to a smaller extent, also in peripheral tissues (for review see Newell-Price, 2003). In normal human pituitary, *POMC* is only expressed in the corticotrophs of the anterior lobe. In mammals that possess an intermediate lobe, *POMC* expression is also found in melanotrophs of this lobe. Tissue-specific expression of *POMC* depends on transcription factors expressed in the tissue. For the corticotroph lineage in the pituitary, T box factor TPIT is needed, acting in synergy with PTX1 to drive corticotroph-specific *POMC* expression (Maira et al., 2003; Newell-

Price, 2003; Pulichino et al., 2004). After being translated, full length POMC undergoes posttranslational processing, in which proteolysis takes place, followed by proper modification of newly cleaved peptides by glycosylation, amidation, or acetylation (Eipper and Mains, 1980). Proteolytic cleavage taking place at certain dibasic sites is carried out by tissue-specific prohormone convertases. Best known are the convertases PC1 and PC2 (Benjannet et al., 1991; Seidah et al., 1992). Human POMC contains eight pairs and one quadruplet of basic amino acids, which are potential cleavage sites of processing enzymes. The expression of prohormone convertase (PCs) and the cleavage sites used are tissue specific. In the corticotrophs of anterior pituitary, only PC1 is present with limited proteolytic action, thus only four of these cleavage sites are used generating a total of six peptides: N-POMC, joining peptide (JP); ACTH, ß-lipotrophin (ß-LPH), γ-LPH and ß-endorphin (ß-end) and ACTH is the main end-product (Fig. 5). In the melanotrophs, the presence of both PC1 and PC2 and their coordinate actions lead to a more pronounced proteolysis using all cleavage sites resulting in smaller fragments: γ-MSH (cleaved from N-POMC), α-MSH, CLIP (from ACTH), ß-MSH, ß end 1-31, ß-end 1-27 (from ß-LPH) (for review see Raffin-Sanson et al., 2003).

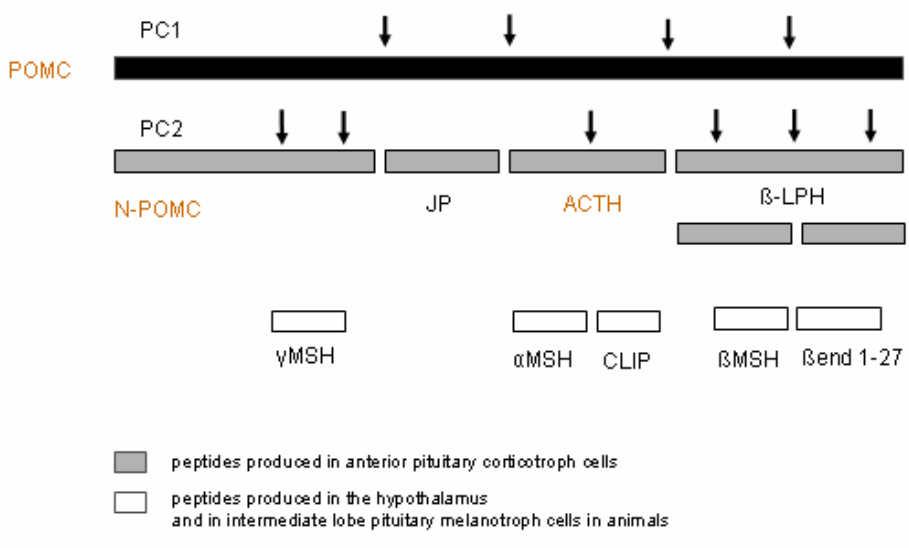

Fig. 5. POMC processing and POMC derived peptides. PC1, PC2: prohormone convertase 1 and 2. Arrowheads indicate potential cleavage sites for processing enzymes. ACTH is the main product of POMC processing in the anterior pituitary. CLIP: corticotrophin like intermediate lobe peptide. JP: joining peptide (taken and modified from Raffin-Sanson et al., 2003).

POMC derived melanocortin peptides bind with different affinity and specificity to a set of five homologous melanocortin receptors (MCRs), which belong to the G-protein-coupled receptor family (Mountjoy et al., 1992; Wikberg, 1999). The bioactive end-products of POMC processing are therefore varied in different tissues. The main product in the hypothalamus is α-MSH that binds to MC3 and MC4 receptors to regulate metabolic pathways and food intake. The pituitary corticotrophs produce predominantly ACTH which acts on MC2 receptors and regulates adrenal function. MSH peptides bind to MC1R in the skin to influence pigmentation (Logan et al., 2003).
Fig. 5 shows a diagram of peptides from human POMC. With respect to multiple biological effects of POMC derived peptides this thesis focusses on the role of POMC on adrenal function and development.

1.6.2 ACTH and adrenal function

ACTH is regarded as the major POMC derived peptide hormone secreted by corticotrophs in the anterior pituitary and a well known regulator of adrenal function. It acts on the adrenocortical cells via binding to its receptor (ACTH or MC2 receptor (MC2R)) on the surface of the cells. The MC2R is a G-protein coupled receptor (Mountjoy et al., 1992). Upon binding to ACTH, MC2R activates its associated G-protein, leading to the activation of adenylyl cyclase, causing an increase of intracellular cAMP levels that activates protein kinase A and initiates the cascade of intracellular signalling events (for review see Gallo-Payet and Payet, 2003). Accordingly, cAMP analogs (*e.g*. 8-bromo-cAMP) or substances that increase adenylate cyclase activity (*e.g*. forskolin) are often used to mimic the actions of ACTH (Kramer et al., 1984).

ACTH stimulates steroidogenesis in two fashions: an acute and a chronic fashion (Lehoux et al., 1998; Simpson and Waterman, 1988). In the acute fashion, effects occur within minutes by stimulating the mobilization and transport of cholesterol by steroidogenic acute regulatory protein (StAR) from the lipid droplets to the inner membrane of mitochondria. Thus more substrate becomes available to steroidogenic enzymes for steroid biosynthesis. Accordingly, the acute effects lead to an immediate increase of steroid output, adapting to the increased steroid demand during stress (Lehoux et al., 1998; Simpson and Waterman, 1988). Complementary to the acute effects, the chronic effects of ACTH involve the

stimulation of steroidogenic genes leading to increased levels of steroidogenic enzymes (Chung et al., 1997; Hu et al., 1991; Lehoux et al., 1998). These effects occur more slowly, contributing to the maintenance of the organ to secrete hormones (reviewed by Hu et al., 2001).

However, whether ACTH is also essential for physiological adrenal growth and proliferation remains uncertain. Studies *in vitro* suggest that ACTH mainly act as a differentiation factor lacking mitogenic activity (Zwermann et al., 2005). Recently it has also been demonstrated that exogenous ACTH even inhibits the growth of adrenal tumor cells in a mouse tumor model (Zwermann et al., 2005).

In humans, mutations in the *MC2R* cause familial glucocorticoid deficiency (FGD) called type 1 FGD (Clark et al., 2005). These patients are usually seen in early childhood with high plasma ACTH, low plasma cortisol, normal mineralocorticoids, recurrent hypoglycemia, hyperpigmentation, and increased bodily growth (Fluck et al., 2002). A mouse model deficient in MC2R has not yet been established. As *in vivo* ACTH is always secreted together with other POMC derived peptides, studies on the effects of ACTH alone on adrenal growth and development *in vivo* are most difficult. Therefore, animal models lacking the MC2R are needed to investigate this issue.

1.6.3 N-POMC and adrenal proliferation

The main N-terminal POMC product in humans is 1-76-POMC. This peptide is also named pro-γ-MSH as cleavage of amino acids 51 to 62 from this peptide results in γ-MSH (Estivariz et al., 1992). Since the early 1980's there have been studies showing mitogenic effects of this peptide on adrenocortical cells. It has been reported that adrenocortical growth induced by N-POMC requires proteolysis. Short, extremely N-terminal POMC which do not contain the gamma-MSH-sequence exhibited mitogenic activity in the adrenal of adult rats both after hypophysectomy and after dexamethasone treatment (Estivariz et al., 1982; Estivariz et al., 1988b; Lowry et al., 1984; Lowry et al., 1983), whereas 1-76-POMC peptide did not have a trophic effect on adrenals of hypophysectomized rats (Estivariz et al., 1988b). Moreover, it has been shown that

proliferative activity in adrenals of adult rats could be induced from 1-76-POMC after treatment with trypsin (Estivariz et al., 1982). Studies from Lowry's group characterized a serine protease named adrenal specific protease (Asp) which is specifically expressed in the adrenal cortex and upregulated during adrenal growth. This enzyme could cleave the biologically inactive pro-γ-MSH into a shorter peptide exhibiting mitogenic activity (Bicknell, 2002; Bicknell et al., 2001).

Further analysis showed that the extracted 1-28-POMC was more potent as an adrenal mitogen than synthetic 1-28 POMC (Estivariz et al., 1988b). This difference is most likely due to the secondary structure of 1-28-POMC. This peptide contains 4 cystein residues at the positions of 2,8,20 and 24. Under physiological conditions the cystein residues at positions 8 and 20 as well as 2 and 24 bind to each other via disulfide bridges (Fig. 6), whereas during standard peptide synthesis different disulfide bridges are formed (Bennett et al., 1986).

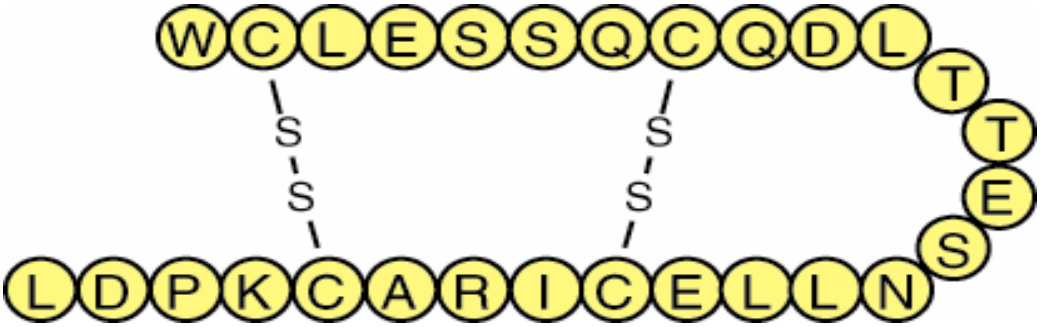

Fig. 6 . Secondary structure of 1-28-POMC with the disulfide bridges between cystein residues 2 and 24, 8 and 24.

The important physiological function of N-POMC is also suggested by the highly conserved amino acid sequence with the characteristic 4 cystein residues in different species (Bennett et al., 1986). Investigation by Ross et al. further demonstrated that infusion of bovine extracted 1-77- POMC led to increased adrenal growth in sheep (Ross et al., 2000). However, the 1-49-N-POMC surprisingly had no effect (Ross et al., 2000). Fassnacht et al. showed proliferative activity of authentic 1-28-POMC in different adrenal

cell lines (Fassnacht et al., 2003). Analysis of signal transduction demonstrated an activation of promitogenic MAP-Kinase/Erk-signal transduction and the protein kinase B pathway. Moreover, N-POMC also demonstrated inhibitory effects on steroid biosynthesis (Fassnacht et al., 2003).

1.6.4 Pituitary/POMC deficiency and adrenal function and development

POMC deficient phenotypes can be observed in patients and animals that harbour inactivating *POMC* gene mutations, in ACTH deficient patients and animals, in *TPIT* gene mutants, in hypophysectomized animals, and in anencephalic fetuses.

POMC deficient patients exhibit adrenal insufficiency with hypocortisolism, lack of zona fasciculata (ZF) and zona reticularis (ZR) with normal zona glomerulosa (ZG) (Krude et al., 2003; Krude et al., 1998; Krude and Gruters, 2000). In addition, they demonstrate early onset of obesity and pigmentation disorders with pale skin and red hair (Krude et al., 2003; Krude et al., 1998; Krude and Gruters, 2000). These features indicate consequences of the lack of ligands for MC2-, MC3- and MC4-, and MC1 receptors, respectively.

The pituitary-restricted transcription factor (TPIT) is a T-box transcription factor important for terminal differentiation of pituitary POMC-expressing corticotrophs and melanotrophs. In human and mice mutations of the *TPIT* gene cause a neonatal-onset form of congenital isolated ACTH deficiency (IAD) (Pulichino et al., 2003). *Tpit* -/- mice have hypoplastic adrenals with most significant loss at the level of glucocorticoid-producing fasciculata layer and undetectable plasma corticosterone accompanied by very low plasma ACTH (Pulichino et al., 2003).

1.6.4.1 Mouse models deficient in POMC

The first mouse strain deficient in all POMC peptides was generated in 1999 by Yaswen et al. using homologous recombination of a replacement-type POMC allele containing a deletion of exon 3 encoding all relevant POMC-derived peptides (Yaswen et al., 1999). This mutant strain was obtained from a mixed white-bellied agouti 129 genetic

background. These *Pomc*-null mice were analysed at six months of age and showed obesity, altered pigmentation and atrophied adrenals, as observed in human patients having a mutation in the *POMC* gene. Concerning the adrenals, these mice had no macroscopically visible adrenal glands with undetectable medullary enzyme gene expression, undectable serum corticosterone and aldosterone (Yaswen et al., 1999). The authors therefore suggested that POMC-derived peptides are essential for adrenal development (Yaswen et al., 1999). Later, 2003, Smart et al. showed that *Pomc*-null mice on a C57BL/6 genetic background have black coat colour and adrenal atrophy (Smart and Low, 2003). In contrast to the mice described by Yaswen et al., these mice showed normal pigmentation, suggesting that POMC peptides are not essential for the production of eumelanin (black/brown) pigmentation (Smart and Low, 2003). *Pomc*-null mutants of both male and female are fertile but the offspring of homozygous parents died perinatally (Smart and Low, 2003). Additionally, daily exogenous ACTH administration could not rescue the adrenal phenotype in these *Pomc*-null mice (Smart and Low, 2003). Further investigations showed that in these mutants, corticotrophs, melanotrophs and POMC neurons were still present but did lack POMC, as expression of GFP-POMC constructs were detected in these cells with normal number and location in GFP-POMC transgenic *Pomc* -/- mice. It is still unclear whether pituitary corticotroph and melanotroph void of POMC peptides retain any physiological function (Smart and Low, 2003). All of these observations suggest that normal development and function of the adrenal gland depend on POMC peptides. Furthermore, in 2004 Coll et al. analysed *Pomc*-null muntants at 3 months of age and demonstrated hypoplastic adrenal glands with distinct cortex and medullary components but a disrupted cortical structure, undetectable serum corticosterone, reduced circulating aldosterone, and an abnormal response of the HPA axis (Coll et al., 2004). Treatment with supraphysiological doses of ACTH restored within 10 days serum corticosterone concentration and adrenal mass of these mutants, as well as cortical architecture. However the gland was restored in a hypertrophic manner, as the mass of a single cell was increased but proliferation markers remained unchanged (Coll et al., 2004). Interestingly, however, a most recent study in these *Pomc*-null mice analysed the animals at birth reporting normal adrenal glands in the mutants (Karpac et al., 2005). Even the homozygous *Pomc*-null mice born to homozygous *Pomc*-null mutant dams also showed normal adrenals at birth suggesting that POMC derived peptides are not required for

prenatal development of adrenal structures in mice (Karpac et al., 2005). However, from two weeks after birth onwards mutant adrenal cells fail to proliferate and mutants develop adrenal atrophy until the adrenals eventually disappear macroscopically several months after birth (Karpac et al., 2005). Plasma corticosterone of these mutants was undetectable, even after exogenous ACTH administration, already at one week after birth (Karpac et al., 2005). In these experiments, ACTH could not induce corticosterone production but transplantation of POMC-null mutant adrenals to adrenalectomized wild-type littermates resulted in adrenals with normal morphology and production of both corticosterone and aldosterone (Karpac et al., 2005). The authors, therefore, concluded that POMC derived peptides are not needed for prenatal adrenal development and that POMC peptides other than ACTH are required for postnatal proliferation and maintenance of adrenal structures and functions (Karpac et al., 2005).

1.6.5 Zebrafish as a model system

Zebrafish (*Danio rerio* or *Brachydanio rerio)*, a teleost belonging to the family of Cyprinidae is a small tropical fresh water fish, native to India and Burma. Recently, it has become an important vertebrate model for a wide spectrum of endocrinological studies (for review see McGonnell and Fowkes, 2006). However, the major use of zebrafish has been in developmental biology. As vertebrate zebrafish shows many experimental advantages of invertebrate models like *drosophila* such as the ease of maintainance under laboratory conditions, the large number of offsprings (100-200 eggs weekly), the external fertilisation, the short development and generation time (3 months). Furthermore, the embryos are transparent, facilitating live imaging and observation of developmental processes as well as molecular and embryonic micromanipulation. DNA, RNA, morpholino oligo injections or cell transplantation are easily performed to the embryos during early development. In addition, many genomic, molecular and histochemical techniques are now established making the use of zebrafish very appealing (Westerfield, 2000). The zebrafish genome is soon expected to be completely sequenced and its data is updated daily at www.ensembl.org/Danio_rerio/index.html.

Short generation time of zebrafish facilitates the search for gene functions via mutagenesis screens in which mutants are generated by N-ethyl-N-nitrosourea (ENU), the most efficient

chemical mutagen for this system. Full-scale mutagenesis screens have generated many mutant fish with various defects for studies on development of many systems (Driever et al., 1996; Haffter and Nusslein-Volhard, 1996; Herzog et al., 2004b; Kim et al., 2006). Furthermore, functions of genes involving early embryonic development can be easily analysed using morpholino strategy. Antisense morpholino technology is used to knockdown the target gene. Morpholinos are antisense oligonucleotides in which the deoxyribose is substituted with an N-morpholin ring. It can therefore bind to the mRNA and block the translation of the target gene with greater stability and fewer non-specific effects than other antisense DNA analogues (Corey and Abrams, 2001; Summerton and Weller, 1997).

With respect to this work that focuses on the interactions between pituitary and interrenal during early embryonic development, zebrafish was thought a suitable model system because of the experimentally established advantages of this model, as outlined above. Steroidogenic pathways in teleosts are similar to that in mammals and cortisol is the main corticosteroid produced by the interrenal organ (see 1.3.2). Steroidogenesis in zebrafish has recently been studied (Hsu et al., 2002; Hsu et al., 2006a; Hsu et al., 2006b; Lai et al., 1998). The main steroidogenic genes such as *cyp11a1* and *3β-hsd* (Lai et al*., 1998)* are also present in zebrafish. Additionally, *star* and *mc2r* genes that can serve as specific interrenal markers were also cloned and characterized (Bauer et al., 2000; Logan et al., 2003), providing excellent tools for the analysis of interrenal development.

Through analysis of four mutants isolated from a screening of F3 offspring of ENU-mutagenized founder fish, zebrafish adenohypophysis development was investigated and shown to share key principles with the development of higher vertebrates (Herzog et al., 2004b; Herzog et al., 2003; Liu et al., 2003a). Lacking different pituitary cell types, these mutants can serve as ideal models for investigating the role of the pituitary in early development of the interrenal organ. These mutants define four genes essential for different steps of adenohypophysis development of which 3 genes have already been indentified: the *lia/fgf3* required for the entire adenohypophysis (Herzog et al., 2004a; Herzog et al., 2004b), the *aa/eya1* required for corticotropes, melanotropes, thyrotropes, and somatotropes, but not lactotropes (Herzog et al., 2004b; Nica et al., 2006), the p*it1*

required for lactotropes, thyrotropes, and somatotropes (Herzog et al., 2004b; Nica et al., 2004), similar to its mouse ortholog.

In addition, zebrafish *ff1b*, the homologue of mammalian stroidogenic factor-1 was isolated and shown to be essential also for interrenal development (Chai and Chan, 2000; Chai et al., 2003; Hsu et al., 2003; von Hofsten et al., 2001). In the *ff1b* knockdown embryo, absence of the interrenal primordium was observed. Moreover, *ff1b* was shown to directly regulate *cyp11a1* expression, as *SF-1* to *CYP11A1* expression in mammals (Hsu et al., 2003).

1.7 Aims of this work

It is well established that the pituitary plays an essential role in human adrenal function. However, the pituitary signals governing adrenal development and growth have not yet been well defined. In particular the respective roles of corticotroph-derived ACTH and other POMC peptides remain uncertain. Moreover, there are conflicting data on the influence of the pituitary gland on adrenal organogenesis in higher vertebrates. As adrenal organogenesis is difficult to study in mammalian models, it is the aim of this work to establish zebrafish as model system to study adrenal (interrenal) organogenesis and to further clarify pituitary adrenal interactions during adrenal development. To this end the work is divided into 3 main steps:

1. Cloning and characterization of the zebrafish *pomc* gene

2. Analysis of the physiological organogenesis of the interrenal gland in wild-type zebrafish embryos with regard to both the steroidogenic and the chromaffin components of the interrenal organ

3. Analysis of interrenal organogenesis in pituitary mutants, in *mc2r*-knockdown embryos and in dexamethasone treated embryos in comparison to wild-type embryos to elucidate the contribution of different pituitary cell types and Pomc-derived peptides on interrenal development, and finally investigation of the development of feedback regulation at the pituitary level.

2 Materials and methods

2.1 Materials

2.1.1 Animals

The wild-type fish embryos were obtained from strains Tü-AB and K-WT maintained at the fish facility of the Department of Physiological Chemistry I, University of Würzburg. The mutants were gained from a zebrafish ENU mutagenesis screen for mutations affecting adenohypophysis development carried out in the Max-Planck-Institute of Immunobiology in Freiburg (Herzog et al., 2004b). The following alleles were used: *aal/eya1*: t22744 (Herzog et al., 2004b; Nica et al., 2006), *lia/fgf3*: t24152 (Herzog et al., 2004a; Herzog et al., 2004b), and pit1: t21379 (Herzog et al., 2004b; Nica et al., 2004).

2.1.2 Competent cells and vectors

The E.coli Top 10- and Top10 F' competent cells were used. These cells were provided with the TOPO TA cloning® kits (Invitrogen GmbH, Karlsruhe, Germany) and are particularly optimized for the transformation with pCR® the II-TOPO® vector. Only small vector quantities are needed for a transformation to receive high colony density. The vector is suitable for the blue/white selection, whereby an induction with IPTG is needed for the E. coli Top 10F'.

2.1.3 Enzymes

The following restriction enzymes were provided by MBI Fermentas (Fermentas GmbH, St. Leon-Rot, Germany):

- BamHI (10 u/µl) 5'-G↓G A T C C-3'
- EcoRI (10 u/µl) 5'-G↓AATTC- 3'
- EcoRV (10 u/µl) 5'-GAT↓ATC-3'
- Hind III (10 u/µl) 5'-A↓A G C T T-3'
- NcoI (10 u/µl) 5'-C↓C A T G G-3'
- XhOI (10 u/µl) 5'-CT↓C G A G-3'

Polymerases used for DNA amplification and RNA *in vitro* transcription:

- HotStarTag™ DNA Polymerase — Qiagen (QIAGEN GmbH, Hilden,Germany)
- Reverse transcriptase — Qiagen(QIAGEN GmbH, Hilden, Germany)
- Sp6 and T7 RNA polymerase — Roche (Roche Diagnostics GmbH, Mannheim, Germany)

Other enzymes:

- DNAse — Roche (Roche, Mannheim, Germany)
- Proteinase K — Sigma (Sigma, Taufkirchen, Germany)
- RNase inhibitor — Roche (Roche, Mannheim, Germany)

2.1.4 Media, buffers and solutions

2.1.4.1 Media and solutions for bacteria culture and TOPO cloning

- LB-Medium (1L):

bacto-tryptone (Peptone 140)	10g
yeast extract	5g
NaCl	10g

The pH of the medium was adjusted to 7.0 using 5 N NaOH. The volume was completed to 1000 ml with dH_2O and autoclaved at 121°C, 15 bar for 20 min.

- LB-Agar- ampicillin plates:

1.5% agar in LB medium: 15g agar was added to 1l LB medium, autoclaved at 121 °C, 15 bar for 20 min, cooled down (using a magnetic stir bar) to 60°C. After adding 2ml ampicillin 50µg/ml, it was plated to about 40 plates (90mm plates). Agar plates were stored inverted at 4°C in the dark.

- S.O.C. medium (as provided with the TOPO-TA cloning kit)

  2% Tryptone

  0.5% Yeast Extract

  10 mM NaCl

  2.5 mM KCl

  10 mM MgCl2

  10 mM MgSO4

  20 mM glucose

- Ampicillin stock solution (50 mg/ml) — 500 mg ampicillin is dissolved in 10 ml dH_2O, and stored at -20°C

- IPTG 1M — IPTG 1M in 50% ethanol, stored at -20°C

- X- Gal 20mg/ml — 200 mg X-Gal in 10 ml dimethylformamide, stored at -20°C

2.1.4.2 Media for culturing zebrafish embryos

- Danieau's blue medium:

  0.1 % NaCl

  0.003% KCl

  0.004% $CaCl_2$ x H_2O

  0.016% $MgSO_4$ x $7H_2O$

  0.0001% methylene blue

- E3 Medium:

  5 mM NaCl

  0.17 mM KCl

  0.33 mM $CaCl_2$

  0.33 mM $MgSO_4$

2.1.4.3 Solutions and buffers for whole mount ISH of zebrafish embryos

- 0.06% Phenylthiourea: (0.06% PTU) — 0.15g PTU (Sigma, P-5272**)** is dissolved in 250ml H_2O, stired under a hood until dissolved, aliquoted and stored at -20°C

- 4% Paraformaldehyde: (4% PFA) — 2g PFA in 50 ml PBS, heated to 70°C, stired for 2h under a hood until dissolved, cooled down to 4°C. 4µl 1N NaOH is added, mixed, and stored at -20°C

- 20% Tween 20: — 10 ml tween 20 in 40 ml dH_2O

- PBS buffer:

  0.8% NaCl
  0.02% KCl
  1.15% Na_2HPO_4
  0.02% KH_2PO_4
  0,01% $MgCl_2$
  0,01% $CaCl_2$
  pH 7.3

- PBST: — 2.5 ml 20% tween 20 were added to 500 ml PBS

- SSC 20x (1l):

  NaCl 175.3g
  Sodium citrate 88.22g
  These were dissolved in 800 ml of dH_2O, adjusted to pH 7 with 1N HCl, dH_2O is added to 1l

- Hybridization solution (Hyb+):

End concentration:		For 40 ml:	
50%	formamide	20 ml	formamide
5x	SSC	10 ml	20x SSC
0.1%	Tween 20 or triton X-100	200µl	20% triton X-100
pH 6	adjusted with 1M citric acid	375µl	citric acid 1M
5mg/ml	Torula yeast RNA	200µl	heparin 10mg/ml
50µg/ml	heparin	400µl	yeast RNA 50mg/ml
		H_2O added to 40ml	

- 1% blocking reagent solution: 1g blocking reagent (Roche) in 100 ml PBST, stir until dissolved and store at -20°C

- 1M Tris/HCl, pH 9.5: 121.4 g Tris in 800 ml dH_2O, stir until dissolved, adjust to pH 9.5 with 1N HCl, add dH_2O to 1l

- 1M $MgCl_2$: 20.3 g ml MgCl2 · 6 H_2O dissolved in 100 ml dH_2O

- 5M NaCl: 146 g NaCl dissolved in 500 ml dH_2O

- Alkaline phosphatase staining buffer (Xpho buffer):

End concentration:		For 40 ml:	
0.1 M	Tris/HCl pH 9.5	4ml	1M Tris/HCl 9.5
50 mM	$MgCl_2$	2ml	1M $MgCl_2$
0.1%	Tween or Triton X-100	200µl	20% Tween 20
0.1M	NaCl	800µl	5M NaCl
		H_2O added to 40 ml	

- 1M glycine/HCl, pH 2.2: 7.5 g glycine dissolved in 70 ml d H_2O, pH adjusted to 2.2 with 1N HCl and H_2O added to 100ml

- 0.1M glycine/HCl, pH 2.2, 0.1% tween: 5 ml 1M glycine/HCl, pH 2.2 and 250µl 20% Tween added to 45 ml dH_2O, vortexed.

2.1.4.4 Histochemical staining solution for 3ß-Hsd (3ß-HSD staining solution)

- 0.1 M phosphate buffer pH 7.2: 8.9 g $Na_2HPO_4 \cdot H_2O$ and 6.804 g KH_2PO_4 dissolved in 500 ml dH_2O

- Add to 20 ml 0.1 M phosphate-buffer, pH 7.2 :

Polyvinylpyrrolidone (PVP Sigma^R , PVP 40)	2.5 g
Etiocholan -3ß-ol-17-one (Sigma^R ,E - 5251**)**	2.0 mg
ß -Nicotineamide adenine dinucleotide (Sigma^R, N-1511)	30.0 mg
Nitroblue tetrazolium (NBT, Fluka ,74030)	20.0 mg
Ethylenediamine tetra-acetic acid (EDTA,Sigma^R , E5134)	50.0 mg
N,N dimethyl formamide (Merck)	0.2 ml

Dissolve and filter with a sterile filter, and store aliquotes at -20°C

2.1.4.5 Buffers for DNA agarose gel electrophoresis and for restriction digestions

-	Joule - Box- buffer 1x (1l)	Tris - base acetic acid EDTA	4.8 g 5.7 ml 0.614 g
-	6x DNA loading buffer: The loading buffer was stored at 4°C	Bromophenol blue Xylene cyanol Sucrose d H_2O added up to 10ml, stored at 4°C	0.01 g 0.02 g 4.00 g
-	Buffer BamHI (*MBI Fermentas)*	Tris-HCl (pH 8.0 at 37°C) $MgCl_2$ KCl Triton X-100 2-mercaptoethanol BSA	10mM 5mM 100mM 0.02% 1mM 0.1mg/ml
-	Buffer EcoRI (*MBI Fermentas)*	Tris-HCl (pH 7.5) MgCl2 NaCl Triton X-100 BSA	50 mM 10 mM 100 mM 0.02 % 0.1 mg/ml
-	Buffer R (red) (*MBI Fermentas)*	Tris-HCl (pH 8.5 at 37°C) $MgCl_2$ KCl BSA	10 mM 10 mM 100 mM 0.1mg/ml
-	Buffer Tango ^{TM} (yellow) (*MBI Fermentas)*	Tris-acetate (pH 7.9 at 37°C) magnesium acetate potassium acetate BSA	33 mM 10 mM 66 mM 0.1 mg/ml

2.1.4.6 Other solution

-	1 mM dexamethasone	56.9 mg water soluble dexamethasone (Sigma, D2915) dissolved in 10 ml H_2O, aliquoted to 1 ml, stored at -20°C

2.1.5 Oligonucleotides

All primer oligonucleotides were obtained from Thermo Electron GmbH, Ulm. Primers are 20-35 nt in length and partly listed in the appendix (see 6).
Morpholino oligos used in this study were supplied by Gene Tools (Gene Tools, LLC, Philomath, USA).

2.1.6 Antibodies

- Anti-Digoxygenin-AP (anti-Dig-AP) (Roche, Cat. No. 11 093 274 910)
- Anti-Fluorescein-AP (anti-fict-AP) (Roche, Cat. No. 1426346)

2.1.7 Alkaline phosphatase substrats

- BM purple AP (Roche, Cat. No 11 442 074 001)
- INT/BCIP AP (Roche, Cat. No 11 681 460 001)

2.1.8 Chemicals

All the chemicals used in this work were obtained from the following manufactures: MBI Fermentas (Fermentas GmbH, St. Leon-Rot, Germany), Roth (Roth GmbH, Oberteuringer, Germany), Sigma, Fluka (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), Merck (Merck KGaA, Darmstadt, Germany), Roche (Roche Diagnostics GmbH, Mannheim, Germany).

2.1.9 Kits

- HotStarTag™ DNA Polymerase Qiagen, 203205
- QIAGen® One Step RT-PCR Kit Qiagen, 210212
- Ultrafree ® -DA for DNA extraction from agarose Millipore, 42600

- Topo TA Cloning® Kit	Invitrogen, 3064725
- QIAprep® Spin Miniprep Kit	Qiagen, 27106
- Oligotex® mRNA Mini Kit	Qiagen, 70022
- SMART™RACE cDNA Amplification Kit	Clontech, K1811-1
- Dig RNA Labeling Kit (Sp6/T7)	Roche, 1175 025 910

2.1.10 Instruments

- Autoclave	Intergra Biosciences, Tecnomara Deutschland GmbH
- Incubator	Memmert
- DriBlock DB20 heating blocks	Techne
- Micro WILD M3B Binocular	Leica
- PCR- Mastercycler Gradient	Eppendorf
- Shaking incubator	Edmund Bühler
- Biophotometer	Eppendorf
- Centrifuge Rotanta 96 RC	Hettich
- Vortexer	Heidulph
- Biofuge fresco centrifuge	Heraeus
- Water bath	Memmert
- UV- Transluminator	IBI
- pH 526 pH - Meter	MultiCal ®
- Joule Box ™ System Gel Electrophorese Apparatus	Stratagene
- SBA 32 Max 120 g, d = 0,0001 g scale	Scaltec
- Ice machine	Scotmann AF 10
- Max 810 g, d = 0,01 g scale	Kern 572
- Microliter pipettes	Eppendorf, Gilson

-	Heating block	Hartenstein
-	Magnetic stir	Hartenstein
-	Refrigerators (-20°C; -80°C)	LIEBHERR, Genheimer
-	Glas capillary	Harvard apparatus *GC 100-10* 1.0mm O.D. x 0.58mm I.D.
-	Vertical Pipette Puller	KOPF$_{TM}$ Model 720
-	Micromanipulator	Leitz
-	Microinjector	Eppendorf Microinjector 5242
-	Microscopy Axioskop MC 80	Zeiss
-	Microscopy Axiophot	Zeiss
-	Camera HC-2000 3CCD	Fujix
-	Micro Hybridization Oven	BELLCO Glass, INC, NJ

2.1.11 Softwares

- Adaptec Toast 3.5.6
- Adobe reader 7.0
- Adobe® Photoshop® CS
- Corel draw 11
- Endnote 8.0
- Image Gauge
- Microsft excel
- Microsoft Word 2002
- Microsoft Photo Editor 3.0
- Microsoft PowerPoint 2002
- Statview
- NCBI (National Center for Biotechnology Information (Gen Bank and Pubmed): http://www.ncbi.nlm.nih.gov/

- Blast search: http://www.ncbi.nlm.nih.gov/BLAST/
- Aligment : clustalW: http://clustalw.genome.ad.jp/
- Reverse-Complement: http://bioinformatics.org/sms/
- SignalP V1.1: (Roche Diagnostics GmbH, Mannheim, Germany) http://www.cbs.dtu.dk/services/SignalP/

2.2 Methods

2.2.1 RNA extraction methods

The total RNA was isolated from tissue of adult fish and from embryos using trizol reagent (Invitrogen, Karlsruhe, Germany). 1ml Trizol reagent is added to 100 mg tissue of adult fish or 40 embryos at certain stages placed in a glass homogenizer; the tissue or embryos are homogenated. After transferring the homogenate into a new 1.5 ml tube, 200µl chloroform is added and the sample is shaken vigorously for 30 sec and then centrifuged for 5 min at 13000 rpm. Following centrifugation, the mixture is separated into 3 phases: a low red phenol-chloroform phase, a white interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper phase is carefully transferred into a new 1.5 ml tube and 500 µl chloroform is added, the tube is shaken vigorously and the sample is centrifuged once more for 1 min, at 13000 rpm. This additional step is needed for complete separation of protein and genomic DNA from RNA. The aqueous phase is transferred again to a new 1.5 ml-tube and 1ml isopropanol is added; the tube is shaken for 10 sec and the sample is centrifuged for 20 min, at 13000 rpm to precipitate the RNA. The supernatant is then carefully removed. The RNA pellet is washed twice by adding 1 ml 70% ethanol, centrifuging for 1 min each and then dried briefly (it is important not to let the RNA pellet dry completely as this will greatly decrease its solubility). The RNA pellet is dissolved in 30µl RNase free dH_20; its concentration is measured by a photometer with the wavelength of 260 nm and the RNA solution is stored at -70°C.

2.2.2 Poly A+ RNA isolation from total RNA

Poly A^+ RNA is used for 5' and 3' RACE PCR. Poly A^+ RNA isolation from total RNA was performed using Oligotex® mRNA Mini Kits (Qiagen) following manufacture's

instructions. Oligotex resin consists of polystyrene-latex particles of uniform size (1.1 µm diameter) and a spherical shape with dC10T30 oligonucleotides covalently linked to the surface. The particles form a stable suspension that provides a large surface area for rapid and efficient binding of polyadenylic acids. The high capacity and accuracy of hybridization provides high purification of poly A+ mRNA through fast and efficient hybridization. Purified mRNA is then obtained by washing and eluting mRNA from Oligotex. Detailed steps are described in the protocol of the kit and are not repeated here.

2.2.3 DNA amplification by polymerase chain reaction (PCR)

2.2.3.1 RT-PCR

RT-PCR was performed on total RNA using QIAGen® one step RT-PCR kit. 1µg of total RNA of adult fish or of staged embryos was used as template. GSP1 and GSP2 (see appendix 6.1) were the primers used for amplification and cloning *pomc* cDNA. For analysing *pomc* expression of staged embryos, GSP3 and GSP4 were used (see appendix 6.2). These primers span an 1.5 kb- intron. ß-actin cDNA was amplified with GSP7, GSP8 (see appendix 6.2) for control. RT-PCRs were carried out in Mastercycler Gradient (Eppendorf).

Reaction components:

x µl	template-total RNA (~ 1µg)
6 µl	primer mix
10 µl	RT-PCR 5x buffer
10 µl	Q solution
2 µl	10 mM d NTP's
2 µl	enzyme mix (reverse transcriptase and hotstarTag DNA polymerase)
y µl	dd H2O
50 µl	end volume

The RT-PCR program is the following:

1x:	
Reverse transcription	50°C 30:00 min
Inactivation of reverse transcriptase and activation of taq-polymerase	
35 x:	
Denaturation	94 °C 1 :00 min
Annealing	60°C 1 :00 min
Elongation	72°C 2 :00 min
1x:	
Final extension	72°C 10:00 min
Conservation	10°C

RT-PCR products were analysed by agarose electrophoresis using 1-1.5% edithiumbromide stained agarose gel. Negative controls for RT-PCR were also performed for the study of *pomc* expression in zebrafish embryonic development using H_2O and genomic DNA as template.

2.2.3.2 RACE-PCR

Smart TM RACE cDNA amplification kit (Clontech, Alameda, USA) was used to amplify 5' and 3' ends of a cDNA based on its known internal sequence. Gene specific primers (GSP) that hybridize the known internal sequences were applied to amplify the ends of cDNA. Firstly, poly-A^+ RNA was used as template for amplifying 5'- and 3' RACE first strands, whereby "Smart-Oligo" (see appendix 6.1.2) was used for 5'-RACE first strand synthesis. The reactions were set up as following:

For 5' Ready cDNA

2 µl	Poly A^+ RNA (~ 1µg)
1 µl	5'-CDS Primer
1 µl	SMART II A Oligo
x µl	H_2O
5 µl	end volume

For 3' Ready cDNA

2 µl	Poly A^+ RNA (~ 1µg)
1 µl	3'-CDS Primer
x µl	H_2O
5µl	end volume

Each reaction mixture was incubated at 70°C for 2 min and cooled down by placing it on ice for 2 min. Further steps were carried out as instructed in the manufacturer's protocol. 5'- and 3'- Ready cDNA as templates, as well as gene specific primers GSP3 and GSP4 (see appendix) and an anchored primer (UPM, Long Universal Primer, see appendix) were then used to make 5'- and 3' RACE-PCR. A master mix for 5'- and 3' RACE-PCR were prepared as following:

34.5 µl	PCR-grade water
5.0 µl	10x Advantage 2 PCR buffer
1.0 µl	dNTP Mix (10mM)
1.0 µl	50x Advantage 2 Polymerase Mix
41.5 µl	end volume

5'- and 3' RACE-PCR reactions:

For 5' - RACE PCR:

2.5 µl	5'RACE-Ready cDNA
5.0 µl	UPM (10x)
1.0 µl	GSP3 (10 µM)
41.5 µl	Master Mix
x µl	H_2O
50.0 µl	end volume

For 3' - RACE PCR:

2.5 µl	3'RACE-Ready cDNA
5.0 µl	UPM (10X)
1.0 µl	GSP4 (10 µM)
41.5 µl	Master Mix
x µl	H_2O
50.0 µl	end volume

Because the melting temperatures of gene specific primers GSP3 and GSP4 are above 70°C, a touch down PCR was performed with the following program:

5x	94°C	5 sec
	72°C	3:00 min
5x	94°C	5 sec
	70°C	10 sec
	72°C	3:00 min
20x	94°C	5 sec
	68°C	10 sec
	72°C	3:00 min

The amplified fragments were then cloned and sequenced. From these full 5' - and 3' ends of zebrafish *pomc* cDNA that have an overlapping sequence in the *pomc* cDNA the complete POMC cDNA was deduced.

2.2.4 DNA agarose electrophoresis

Agarose gel electrophoresis was carried out to check the progression of a restriction enzyme digestion, to determine the yield and purity of a DNA isolation or PCR reaction and to size-fractionate DNA molecules, which then could be eluted from the gel. Agarose was cooked in 1x Joule-Box-buffer, cooled down to 70°C, then ethidiumbromide was added to a final concentration of 1µg/ml. DNA samples were mixed with 6x loading buffer to a final concentration of 1x loading buffer and run in 1x Joule-Box-buffer. The DNA runs from negative to positive electrode. The running time was about 15-25 min at 100V. DNA bands are visible by exposure to UV light and photos were taken.

2.2.5 DNA extraction from agarose gel

DNA fragments were isolated and purified from agarose gel using Ultrafree®-DA kit (Milian AG, Basel, Switzerland) for further cloning and sequencing. The desired DNA band was cut from agarose gel using a clean scalpel, placed into Millipore tubes and centrifuged for 15 min at 7500 rpm. Under centrifugation-compression DNA is extruded from the gel pores, runs through the pores of the membrane of the filter and is collected in a collection tube.

2.2.6 Phenol/chloroform extraction for cleaning linearized plasmid DNA

Using phenol-chloroform extraction protein can be removed from DNA solutions. This protocol was applied for cleaning linearized plasmid DNA used as template for the *in vitro* transcription of probes used for ISH:

Added to 100 µl digestion reaction: 10µl (1/10 volume) NaAc 3M, pH 5.2, 100 µl (1 volume) phenol. The suspension was then mixed by vortexing and centrifuged at 14.000 rpm for 5 min. After transferring the supernatant to a new tube, 100 µl chloroform was supplemented, mixed and centrifuged at 13.000 rpm for 5 min. The supernatant was transferred into a new tube and mixed with 300 µl 100% ethanol chilled at -20°C. The tube was kept at -20°C for 20 min. The DNA pellet was formed by centrifuging at 13.000 rpm at 4°C for 20 min and resuspended in 25 µl dH_2O.

2.2.7 Measurement of DNA/RNA concentration

Concentration of nucleic acid was determined via measuring optical density using biophotometer (Eppendorf) with a wavelength of 260 nm.

2.2.8 TOPO TA cloning

TOPO TA cloning kits (Invitrogen) can be used to clone PCR products with single 3' adenine overhangs (as amplified by Tag polymerase). The linearized plasmid vector pCR®II-TOPO® has single, overhang 3' deoxythymidine (T) residues that bound covalently to topoisomerase I. Topoisomerase is released after the ligation of the inserts. TOPO TA- cloning® is a highly efficient, one-step cloning procedure that requires no ligase, post-PCR processing, or PCR primers containing specific sequences.

PCR products were mixed with pCR® II-TOPO® vector and salt solution provided with the kit and incubated at room temperature (22-23°C) for 5-30 min (TOPO cloning reaction). Further steps were carried out following the protocols provided by the manufacturer and are not described here. Plasmid-DNA was isolated using QIAprep® Spin Miniprep Kit

(Qiagen) (see 2.2.9). Positive colonies were screened by restriction digestion with ECoRI (see 2.2.10) and sequencing.

2.2.9 Plasmid mini-preparation

Preparation of plasmid DNA was performed using QIAprep® Spin Miniprep Kit (Qiagen). The method is based on alkaline lysis of bacterial cells. In a certain high pH range (pH 12-12.5) linear DNA is denatured, while circular covalently closed DNA remains in solution. The DNA solution is exposed only to an alkaline pH and neutralized afterwards again, while genomic DNA precipitates as insoluble network in the solution. This precipitate and the protein complexes can be removed by centrifuging. RNA is digested by RNase A. A Qiagen column is an anion exchange column. It contains large porous silica-gel balls coated by hydrophilic substances. With suitable buffers plasmid-DNA binds to the column and then is isolated by washing the column. Subsequently, highly purified plasmid is eluted. There are 3 main steps in the protocol:

- **Preparation and clearance of bacterial lysate**

A single bacterial colony is picked and cultured overnight at 37°C in 10 ml culture medium (LB/Ampicilin) in a shaking incubator at about 200 Mot/min. The next morning the cells are harvested by centrifuging 3000 rpm at 4°C for 20 min. The bacterial pellet is resuspended in 250µl buffer P1 containing RNase and then transferred to a 1.5 ml Eppendorf tube. 250µl lysis buffer P2 containing NaOH-SDS is added to the tube and mixed gently by converting the tube. SDS solubilizes phospholipids and protein components of the cell membrane, leading to lysis and release of the cell contents. NaOH denaturates the chromosomal and plasmid-DNA, as well as proteins. The optimized lysis time allows maximum release of plasmid DNA from the cell without release of cell-wall-bound chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline condition may cause irreversibly denatured. The lysis reaction time therefore should not exceed 5 min. The lysate is then neutralized by addition of 350 µl neutralized buffer P3 containing acidic potassium acetate. High salt concentration causes potassium dodecyl sulphate (KDS*) to precipitate and denatured proteins, chromosomal DNA, and circular ebris becomes trapped in salt-detergent

complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution. Since any SDS remaining in the lysate will inhibit binding of DNA to Qiagen resin, the solution is thoroughly but gently mixed to ensure complete precipitation of the detergent.

- **Absorption of DNA to QIAprep-membrance**

The bacterial lysate is then cleared by centrifugation for 10 min at maximum (max) speed. The supernatant is loaded to Qiagen-tip and centrifuged at max speed for 1 min. Under high salt conditions, silicate-membrane absorbs plasmid-DNA selectively and plasmid-DNA is released and eluted by low salt buffer.

- **Washing and eluting plasmid-DNA**

Qiagen-tip is then washed by adding 0.75 ml wash buffer (buffer PE) and centrifuging for 1 min, the flow-through is discarded and the column is centrifuged once more to remove residual wash buffer. Plasmid-DNA is then eluted by placing the QIAprep column in a clean 1.5-ml-Eppendorf tube and adding 50 µl dH_2O or buffer EB to the center of the column, letting it stand for 1 min and centrifuging for 1 min. The concentration of plasmid-DNA is measured by a photometer at 260 nm, normally giving a yield of 150-300ng/µl.

2.2.10 DNA restriction digestion

All restriction enzymes used were from MBI Fermentas (Fermentas GmbH, St. Leon-Rot, Germany). For screening positive colonies gained from TOPO cloning, plasmids were digested with ECoRI in a 10µl reaction. It is important that the digestion reaction volume should at least exceed 10 fold the volume of the enzyme used and that 10U enzyme are used for digestion of 1 µg DNA:

1 µl (plasmid) DNA (200-500ng)
1 µl EcoRI buffer 10x
1 µl ECoRI (10U/µl)

7 µl dd H_2O

10 µl end volume

To linearize plasmids for antisense probe synthese, corresponding enzyme was used depending on the orientation of the insert in the vector, so that reversed sense strand is prior to the promoter for the *in vitro* transcription. For this application, 10µg plasmid was normally linearized in 100 µl reaction.

2.2.11 DNA sequencing

Sequencing of cloned DNA was performed by Medigenomix (Medigenomix GmbH, Martinsried, Germany).

2.2.12 Cryopreservation of bacteria

0.5 ml sterile glycerine was added to 1 ml overnight culture of a single bacterial colony in a 1.5 ml tube. The tube was let at room temperature for 30 min and then kept at -80°C.

2.2.13 Zebrafish whole mount *in situ* hybridization

RNA *in situ* hybridization is used to detect gene expression at the transcriptional level using *in vitro* transcribed antisense mRNAs of genes of interest as probes. The antisense probes containing labelled nucleotides bind to complementary endogenous sense mRNA strands, and therefore can be detected by immunostaining using antibody directed against the labelled nucleotides.

2.2.13.1 Preparation of riboprobes (antisense RNA) for *in situ* hybridization

Digoxigenin (Dig) and fluorescein (Fitc) labelled antisense RNA probes were used for whole mount *in situ* hybridization in this work. Partial cDNAs of zebrafish *ff1b, cyp11a1, star*, *mc2r,* and *"pomc-like*" were amplified by RT-PCR with total RNA from adult zebrafish using the one-step RT-PCR kit (QIAGEN) (see 2.2.3.1). Primers were designed based on the sequences in Genbank for *ff1b* (accession No AF1980868), *cyp11a1* (AF5277558)*, star* (BC075967)*, mc2r* (AY1618489)*,* and *"pomc-like*" (AL845420) and are listed with corresponding annealing temperatures in Tab. 1. PCR fragments were

cloned into pCRII-TOPO vector (see 2.2.8). The dopamine beta hydroxylase *(dβh)* plasmid was kindly provided by Prof. Korzh, IMCB, Singapore. *pit1* plasmid was kindly provided by Dr. Hammerschmidt (Nica et al., 2004). Plasmids were linearized with appropriate restriction enzyme in 100 µl digestion reaction (see 2.2.10) lasting overnight. For the antisense RNA probe, restriction enzyme was chosen according to the orientation of the cDNA insert in the vector so that the reversed sense sequence is placed prior to the promoter for *in vitro* transcription. Following restriction enzymes and RNA polymerase were used to prepare digoxigenin or fluorescein labelled antisense RNA probes: *ff1b* (XhOI/Sp6), *cyp11a1* (HindIII/T7), *star* (XhOI/Sp6), *mc2r* (HindIII/T7), *dβh* (NcoI/Sp6), and *pit1* (NotI/Sp6), "*pomc-like"* (XhOI/SP6) using the RNA labelling kit (Roche). Probes were maintained in 50% formamide and stored at -20°C.

Tab. 1 : primers and annealing temperatures (Ta) used for cloning cDNA of zebrafish *ff1b, cyp11a1, mc2r*, and *star*

Genes	Forward primers (5'-3')	Reverse primers (5'-3')	Ta (°C)
ff1b	ACGGGACGCTGCTGTCGGG	GCTGTCCACCTGCAGCAGC	65
cyp11a1	GCTGCAGGTCAAGGTGACTCGCTCAG	GAGCCAGTTCATAAAGCGTCCACAGCA	60
mc2r	AGCATCCACACTGACTGCGCTGAGGT	GAGCCAGTTCATAAAGCGTCCACAGCA	65
star	GTGTGCTGGCATTTCTTACAGACACATGAGA	GGAAGGTGTGTGCCCTTGTTAAGGC	58
"pomc-like"	GTTCTGTCCGTCTTGGCTTT	GTGAACTGCTGTCCATTGCC	56

In vitro transcription for antisense RNA probes was carried out as following:

For an *in vitro* reaction of a final volume of 20 µl: in 1.5 ml RNAase free tube:

x µl of the linearized template (~ 1-2 µg), H_2O added to 13 µl

2 µl 10 x transcription buffer

2 µl Dig or fitc RNA labelling mix

1 µl RNase inhibitor

2 µl T7 or Sp6 RNA polymerase, depending on the orientation the insert

---

20 µl End volume

The sample was gently mixed and incubated for 2h at 37°C in an incubator in a water bath. After 2 hours of transcription, DNase was added to digest the remaining DNA templates: 2 µl 10x transcription buffer, 17 µl H_2O and 1 µl DNase were added to the tube and the mixture was incubated at 37 °C for 30 min. The newly transcribed RNA was then precipitated with ammonium acetate (NH_4Ac) and absolute ethanol by adding 20 µl 7.8M NH_4Ac (1/2 volume) and 120 µl (3 volumes) of 100% ethanol to the tube and incubating the mix at RT for 50 min for Dig-labelled probe or 25 min for Fitc labelled probe. After centrifugation for 20 min (13000 rpm) at RT, the pellet was washed by centrifuging in 1 ml 70% ethanol for 10 min and dried. Pellet was dissolved in 27 µl RNase-free water. 1 µl was analysed on a 1% agarose gel, 1 µl was used for measuring the concentration of the probe that shows normally 100-300ng/µl. The remaining 25 µl were mixed with 25µl hybridization solution (Hyp+) and stored at -20°C.

2.2.13.2 Zebrafish whole mount *in situ* hybridization (ISH)

All solutions and buffers used for ISH are described in 2.1.4.3.

Embryos were treated with phenylthiocarbamide (1-phenyl-2-thiourea, PTU, Sigma) (see 2.2.17) to suppress pigmentation (Elsalini and Rohr, 2003; Westerfield, 2000). Dechorionated embryos or larva from 20 hpf to 7 dpf were fixed overnight in 4% paraformadehyde in PBS (PFA/PBS) at 4°C and transferred to vials filled with 100% methanol. The methanol was renewed after 5 min and the vials were stored at -20°C at least overnight or longer (several months to a year) until further procession. All further steps were carried out at room temperature (RT) unless otherwise stated. The embryos were placed in 1.5-ml-Eppendorf tubes and washed 4 times, 5 min each in PBST and treated with proteinase K using 2mg/ml proteinase K stock solution to remove the protein around target sequences. The actual time for digestion was varied as shown in Tab. 3 (see appendix 6.3), depending on the embryonic/larva stage. After a brief rinse with PBST, the embryos were further washed for 5 min in PBST and fixed for 20-30 min with 4% PFA/ PBS. After this fixation, the embryos were washed 5 times, 5 min each with PBST and then with 300 µl hybridization solution (Hyp^+) (see 2.1.4.3). 300 µl HYP^+ were then added to the embryos and embryos were prehybridized at 60°C for 2-3 h.

After prehybridization, HYP $^{+}$ solution was removed as much as possible without letting the embryos come in contact with air. 50-100µl of fresh HYP $^{+}$ containing 20-100 ng digoxigenin or fluorescein labelled RNA were added to the embryos and the hybridization was let overnight at 60°C. Hybridization temperature can be adjusted from 50-70°C, according to the specificity of the signal. Next day the probe was removed and the embryos were rinsed shortly with HYP^{+} and washed at 60°C for 15 min each in 3:1, 1:1, and 1:3 (HYP^{+}:2xSSC). The embryos were then rinsed shortly in 2xSSC and washed twice at 60°C in 0.2xSSC/0.1% Triton X-100 for 30 min each. After further washes with PBST (6 times, 5 min each), embryos were blocked for 2 h in 1% blocking reagent in PBST (1%BL, see 2.1.4.3). For use alkaline-phosphatase coupled anti-Digoxygenin and alkaline-phosphatase coupled anti-Fluorescein (anti-Fitc-AP) (see 2.1.6) need to be diluted at 1:6000 and 1:3000, respectively in 1%BL). After blocking, blocking solution was removed from the embryos and 500µl diluted anti-Dig-AP or anti-Fitc-AP were added to the embryos and incubated at RT for 2 h. Following this incubation, the embryos were washed several times with PBST and let overnight at 4°C in PBST. Next day, after further washes for 1-2 h with 15-20 min interval in PBST and 2 washes 10 min each with AP-staining buffer (see 2.1.4.3), the embryos were transferred to a 24-well-microtiter plate and washed once more for 5 min with AP-staining buffer. Depending on the colour desired, BM purple substrate (blue) or INT/BCIP (red) should be used to visualize the signals (the blue signal is stronger and more stable than the red one). The substrates stored at 4°C with needed amount (BM purple with 500µl/ well; 7.5µl INT/BCIP concentrate is diluted in 1 ml AP staining buffer for use of 500 µl/well) were taken out to RT 30 min before use. After the last wash with AP staining buffer, 500µl BM purple or diluted INT/BCIP were added to a well containing the embryos for detection of the signals. The reaction was incubated at RT or at 4°C when lasting overnight, observed under binocular and stopped with PBST when the signal is satisfactory. The specimen were then washed several times with PBST and fixed 30 min at RT or overnight at 4°C in 4%PFA/PBS, followed by several washes with PBST again. BM-purple- or INT/BCIP stained embryos were kept in benzyl alcohol/benzyl benzoate (2:1) or 80% glycerol in PBST, respectively at 4°C.

For double *in situ* hybridization, a mix of digoxigenin labelled and fluorescein labelled probes was used for hybridization. After the incubation of 1st antibody (anti - digoxigenin

AP) and the detection of the 1st signal in blue colour by BM purple substrate, the embryos were incubated 2 times, 15 min each in 0.1M Glycin/HCl, pH2.2/0.1%Tween to completely remove the activities of the AP coupled to 1st antibody, followed by 4 times washes, 5 min each with PBST and 2-hour-incubation with 2nd antibody (anti-fluorescein AP, diluted to 1:3000 in 1% blocking reagent in PBST). After a 2-hour-wash with PBST and 3 washes in AP-reaction buffer, 10 min each, the other AP staining substrate that could give different colour (red, INT/BCIP substrate solution that was diluted 7.5µl/ml in AP staining buffer) was added to detect the 2nd signal. As the red colour induced by INT/BCIP is unstable in alcohol, double-stained embryos were kept in 80% glycerol in PBST. The embryo was mounted in benzyl alcohol/glycerol (5:1) to take photos. In this mounting solution, only the blue stain is stable, whereas the red fades out within minutes. To confirm whether individual cells are positive for one probe only (*red*) or for both probes (*red* and *blue*), photos were taken right after mounting and after the *red* had vanished.

2.2.14 Densitometry and statistics for *in situ* hybridization signals

In situ hybridized embryos were manually dissected from the yolk and flat mounted in benzyl alcohol/benzyl benzoate (2:1) for BM-purple stained ones or in benzyl alcohol-glycerol (5:1) for INT/BCIP- or INT/BCIP/BM- purple stained ones for taking photos. For comparison photos of embryos from the respective groups were always taken in the identical orientation and illumination using the same magnification. Areas and density of the respective signal was measured by the Image Gauge program, Version 3.4 (FULJI PHOTO FILM Co, LTD).

Significance of differences was evaluated by ANOVA using the statistical software program Stat View 4.51. A value of $p < 0.05$ was considered statistically significant with post-hoc analysis carried out by Fisher-PLSD test. All results are expressed as means ± S.E.M.

2.2.15 Chromogenic histochemical staining for 3ß-Hsd

Whole embryos were histochemically stained for 3ß-Hsd enzymatic activity using a protocol based on Levy's method as previously described (Chai et al., 2003; Grassi Milano et al., 1997). In the presence of etiocholan-3ß-ol-17-one, 3ß-Hsd produces an insoluble

diformazan precipitate by transfering protons to a proton acceptor such as tetrazolium salts. Therefore, nitroblue tetrazolium (NBT), a colour substrate was used for the specific detection of 3β-Hsd activity in adrenal/interrenal tissue (Chai et al., 2003; Grassi Milano et al., 1997). After overnight fixation in 4% paraformaldehyde in phosphate buffered saline (4% FFA/PBS), embryos were washed twice with 0.1% Tween in PBS (PBST). The chromogenic reaction was perfomed at 37°C in 3ß-HSD staining solution (see 2.1.4.4) containing etiocholan-3b-ol-17-one as substrate. Reactions were monitored until sufficient signal intensities were obtained (3-8 h, depending on embryonic/larval stage). Staining reactions were terminated by washing the embryos in PBST. The embryos were then fixed in 4% PFA/PBS for 30 min-1h. Specificity of the staining was checked by staining the embryos in the staining solution with all chemical components contained in 3ß-Hsd staining solution except the substrate etiocholan-3ß-ol-17-one.

2.2.16 Culturing of zebrafish embryos

Adult zebrafish were maintained according to Westerfield (Westerfield 2000) in the fish facility of physiology chemistry I, Bio-centre of the University of Wuerzburg. The mutant fish lines were raised at fish facility of Max-Planck Institute for Immunology, Freiburg. The fish were sex-separated maintained in aquaria lighted up with defined light-dark cycle (10 h light off, 14 h light on). In the afternoon before the day on which the embryos are needed, female and male fishes were placed together in a mating box (normally 8 females and 6 males), a small plastic box that has 2 bottoms: an netted-upper bottom that allow the freshly laid eggs to go through and sink to the lower bottom to avoid eaten by the parent fishes. The eggs were laid and inseminated after the light was switched on. About 100 -200 eggs could be laid by a female fish per time. These eggs then fertilized by the male fish and sink to the bottom of the box. The eggs then transferred by a disposable plastic pipette to a Petri dish (30-60 embryos pro dish) and cultured by embryo medium (Danieau or E3 medium) at 28.5°C and staged according to Kimmel et al (Kimmel et al., 1995). Unfertilized eggs were sorted out at two-cell stage

2.2.17 PTU treatment of zebrafish embryos

Embryos were treated with 0.003% PTU to inhibit pigmentation as described previously (Elsalini and Rohr, 2003; Westerfield, 2000). 1.25 ml of 0.06% PTU stock solution (see

1.1.4.3) was added to a dish containing 24 ml Danieau's medium and about 30-40 embryos at age of 6 to 24 hpf. PTU and medium were changed in every 2 days.

2.2.18 Morpholino injection

Mc2r antisense morpholino was injected into the yolk of 1-2 cell embryos with an optimum concentration of 16.7µg/µl. five-nucleotide-mismatch MC2R morpholino was also injected as control using the identical concentration. Morpholino oligonucleotides were designed and synthesized by Gene Tools, Philomath, OR, USA. Their sequences are as follows: *mc2r* antisense: ATCACTCTTAATTGTAGATCAGTTG, corresponding to nucleotides -12 to -37 in the 5'-UTR of the MC2R cDNA; *mc2r* -mismatch: ATgACTgTTAATTcTAcATgAGTTG.

2.2.19 GFP-based experiments to test morpholino efficacy

To test for morpholino efficiency, a GFP based approach was used as described earlier (Schafer et al., 2005). Briefly, the 25nt target sequence for the *mc2r* morpholino was cloned in front of GFP into the CS2+GFP expression vector using annealed oligos containing BamHI overhangs. Capped mRNA was transcribed *in vitro* using the mMessage mMachine Kit from Ambion (Austin, USA) and injected into zebrafish embryos at the one to two cell stage. Half of the injected embryos were injected with 8ng of the *mc2r* morpholino directly afterwards. Efficiency of morpholino induced knockdown of translation was tested by GFP expression analysis.

2.2.20 Dexamethasone experiments

To analyse feedback regulation of pituitary *pomc* expression by glucocorticoids and expression of steroidogenic genes, wild-type embryos were continuously treated with 40µM dexamethasone, starting at 1 dpf, using water soluble dexamethasone (Sigma). 1ml dexamethasone stock solution (1mM) was added to a dish containing 30-40 PTU treated embryos in 24 ml embryo medium. Medium, PTU and dexamethasone were changed daily and embryos were collected in daily intervals from 2-5 dpf.

3 Results

3.1 The zebrafish *pomc* gene

3.1.1 Cloning of the zebrafish *pomc* cDNA

Because zebrafish and carp belong both to the Cyprinidae family, the published Pomc sequence of the common carp, *C. carpio* (GenBank Accession No. Y14617) was used to perform a BLAST sequence analysis using the public domain NCBI zebrafish HTGS database. A zebrafish draft (access number AL590149.8) that aligned and showed high similarity (more than 82% identity) with carp *pomc* sequence was found and considered as putative zebrafish *pomc* cDNA. Based on this sequence, GSP1 and GSP2 primers that spanned the deduced coding sequence (cds) of the zebrafish *pomc* (Fig. 7) were developed: Forward (GSP1): 5′-CAG AGA TGG TGA GGG GAG TGA GGA TGT TGT GT-3′. Reverse (GSP2): 5′-CTT AAA GCC ACT CAC TCA TCC TTC CTC GGT TG-3′. RT-PCR was performed with total mRNA of adult zebrafish using these primers and 2 fragments of 700 bp and of 2200 bp were amplified. Both fragments were subcloned into pCR® II-TOPO® (Invitrogen, Karlsruhe, Germany) and sequenced. Sequence analysis showed that the 700 bp fragment is the full coding sequence of the zerafish *pomc* cDNA and the 2200 bp fragment is a genomic DNA fragment containing an intron of 1500 bp. Based on the sequence data of the cDNA fragment, RACE-primers were designed: 5′-RACE primer (GSP3) : 5′-TCA CTC ATC CTT CCT CGG TTG GTC TTT ATG CAT TAC GTT-3′, 3′-RACE primer (GSP4): 5′-ATG GCT TTG TCC TTG CCT CCT CGT CCT GCC CT-3′ (Fig. 7). 3′- and 5′-RACE PCR was performed as described in 2.2.3.2 and resulted in one distinct band of 800 bp and 900 bp each, respectively. The fragments were cloned in pCR® II-TOPO® vector and sequenced. The sequence of the full-length cDNA clone was published in GenBank (Accession No. AY125332). Positions of the primers used for cloning full-length zebrafish *pomc* cDNA is shown in Fig. 7.

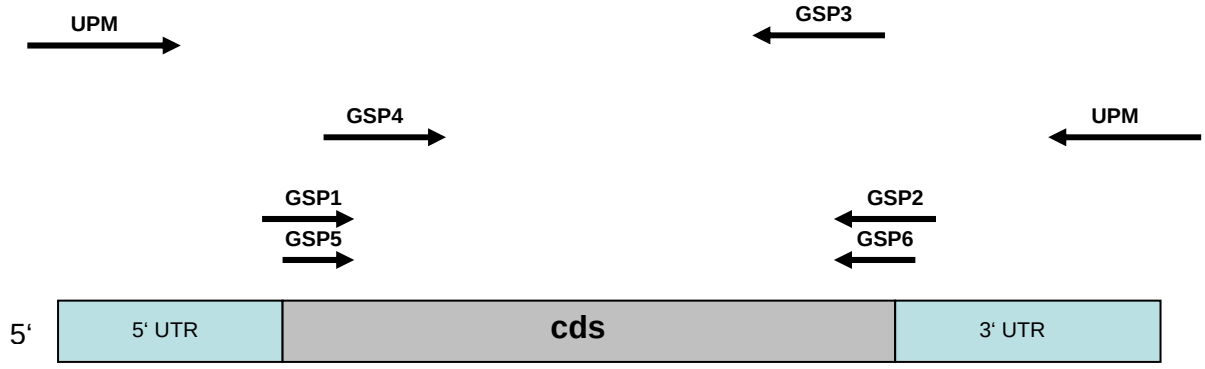

Fig. 7. Position of the primers used for cloning and characterizing zebrafish *pomc* cDNA

3.1.2 Structural features of the zebrafish *pomc* gene

The complete zebrafish cDNA sequence was aligned with the genomic DNA sequence from the HTGS clone RP71-36D5 (GenBank Accession No. AL590149.8) using CLUSTAL W software (http://clustalw.genome.ad.jp/). The alignment showed that the zebrafish *pomc* gene comprises of three exons and two introns (Fig. 8). Both introns start with GT and end with AG (GT/AG rule).

The complete DNA consists of 964 bp and has an open reading frame (ORF) of 669 bp, which codes a polypeptide of 222 amino acids. The deduced amino acid sequence was analyzed (Fig. 8) and the peptide structure was compared with the human POMC (Fig. 9).

Exon 1 has a length of 86 bp and contains the 5′ untranslated region of the *pomc* mRNA. Intron 1 (339 bp) is located in between the 5′-UTR and the coding sequence (cds). Exon 2 (140 bp) encodes the signal peptide and the first part of the N-Pomc. Intron 2 (1522 bp) is located in the N-Pomc-encoding sequence. The main part of coding sequence is located in exon 3 (709 bp) encodes the second part of N-Pomc and the five other peptides (α-Msh, Acth, β-Lipotropin, β-Msh, β-Endorphin), as separated by dibasic cleavage sites

(Benjannet, Rondeau et al. 1991) (Fig. 8 and Fig. 9). Transcription starts 26 bp downstream of a typical TATA box sequence. 3' UTR of the mRNA contains 181 bp. A polyadenylation signal sequence AATAAA is present in this 3′ untranslated region, 161 bp downstream of transcriptional stop codon TGA (Fig. 8).

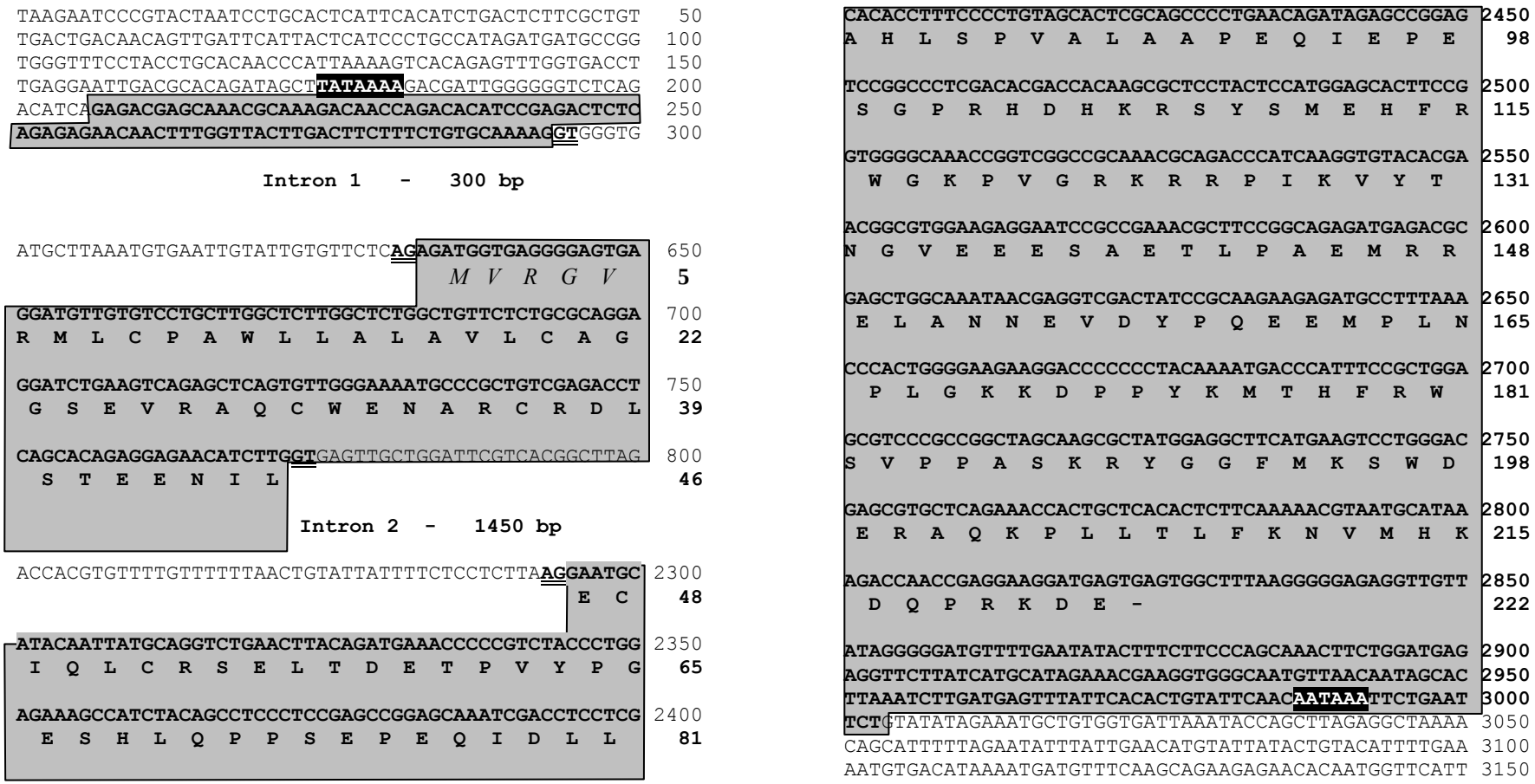

Fig. 8. The *pomc* gene of the zebrafish. Structures of gene, cDNA, and deduced amino acids of zebrafish Pomc. Comparison of the nucleotide sequence of the *pomc* cDNA and the genomic sequence revealed the structure of the *pomc* gene. The three exons are indicated by grey shaded boxes and the intervening sequences are not shown except for the exon/intron boundaries. The exon/intron boundary consensus sequence (GT/AG) is doubly underlined, the TATA box and the poly (A) signal are highlighted with white letters and black boxes. Amino acids are numbered starting from the putative first initiating methionin in exon 2.

3.1.3 Structure of the zebrafish prepropeptide hormone and comparison with other vertebrate POMCs

3.1.3.1 Comparison with the human POMC prepropeptide hormone

Fig. 9 shows the comparison of the amino acid sequence of zebrafish Pomc precursor with the human POMC. The N-terminal hydrophobic signal peptide of zebrafish Pomc contains 28 amino acids whereas the human signal peptide contains 26 amino acids. Four regions of high homology are detected, which in the human POMC represent the N-terminal region (48% amino acid identity human/zebrafish), the ACTH region (78%), β-MSH (69%) and β-endorphin (59%), respectively. Large gaps are found in the zebrafish sequence, when compared to human POMC, in the regions of γ-MSH, hinge peptide, and γ-lipotropin.

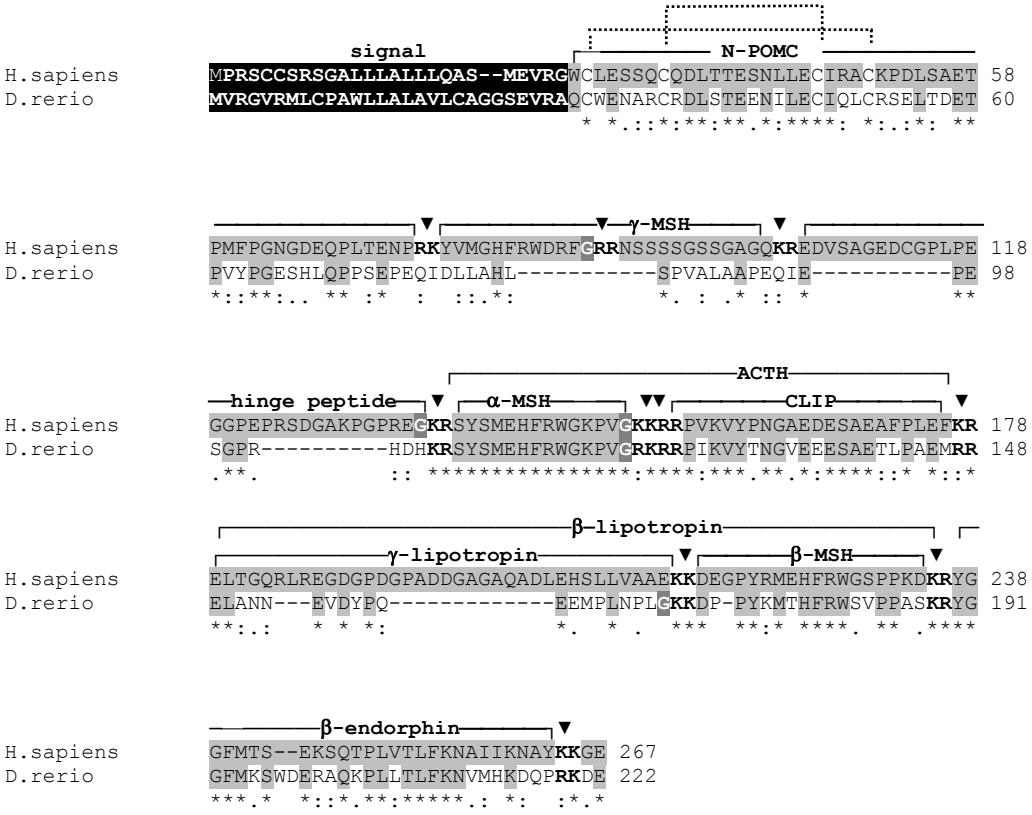

Fig. 9. comparison of the POMC amino acid sequences of man and zebrafish. The designation of the human POMC derived peptide hormones are written above the respective peptides and marked with brackets. The signal peptides are marked with white letters on black ground. The disulfide bonds, established between the conserved cysteine residues, are indicated by dotted lines. Potential dibasic cleavage sites are marked with bold letters and

a black triangle. Completely conserved amino acids are highlighted in grey and marked with *. Similar amino acids are marked with "." resp. ":" and dashes represent gaps.

3.1.3.2 Comparison with POMC in other vertebrates

A multiple sequence alignment of the POMC precursors of several vertebrate species was performed using ClustalW program (http://clustalw.genome.ad.jp/). The Genbank accession No. of the sequences used are : *Cyprinus carpio* I CAA74967, *Cyprinus carpio* II CAA74968, *Danio rerio* AAM93491 *Gallus gallus* BAA34366, *Homo sapiens* PO1189, *Rattus norvegicus* AAM43934, *N. forsteri* AAD37347, *Xenopus laevis* AAA49932 , *M. domestica* AAL13338. The alignment is shown in Fig. 10. As expected, a high homology level was observed between zebrafish Pomc and that of other teleosts (*Cyprinus carpio* I and II). Noteworthy is the absolute conservation of the four cysteine residues in the N-terminal portion of the precursors which form two disulphide bridges (Fig. 10, see also 1.6.3) and the high amino acid identity in the ACTH region (Fig. 10). In all analysed fish Pomc proteins, the γ-Msh and the hinge peptide regions are missing (Fig. 10).

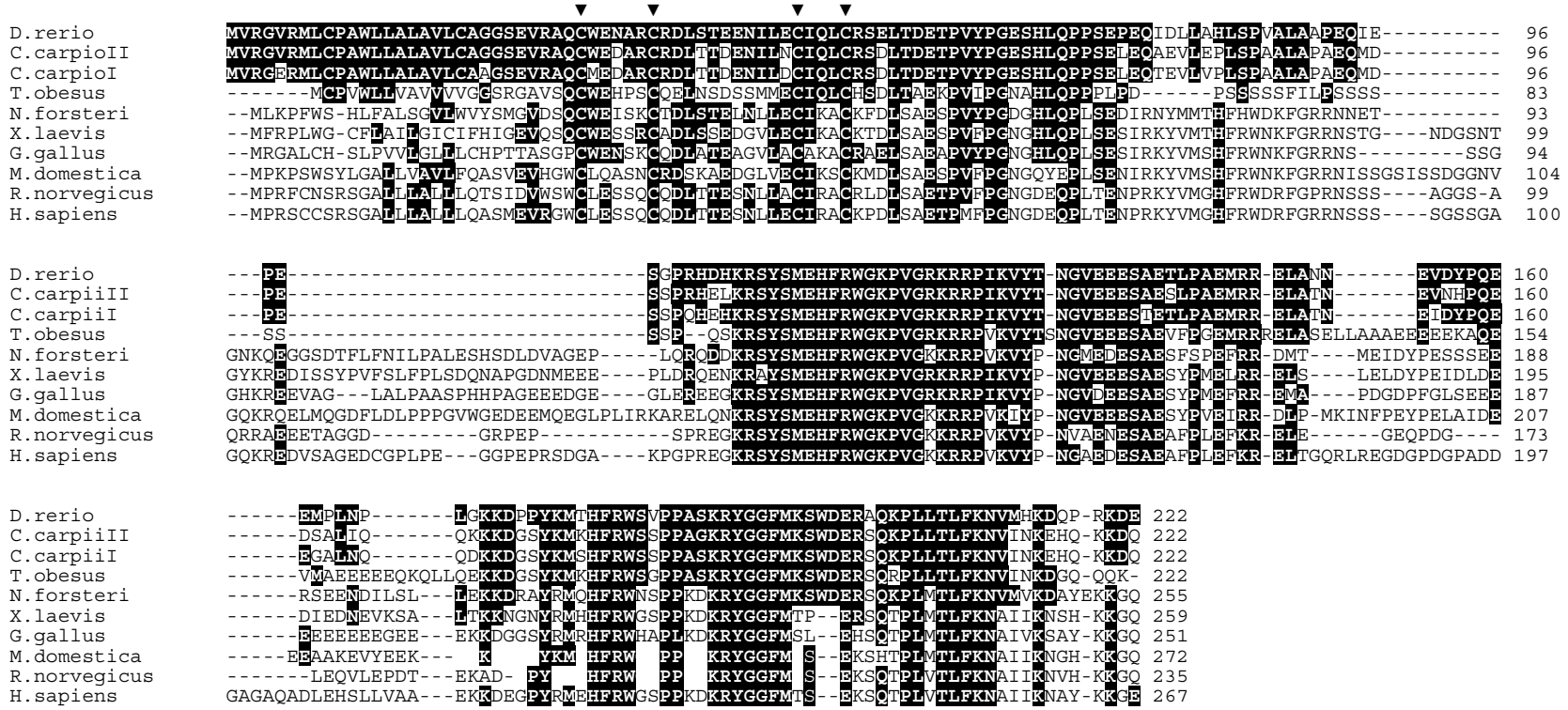

Fig. 10 Multiple sequence alignment of POMC protein sequences of selected species. Identical amino acids are in white letters and black boxed. Conserved cystein residues are marked by black triangles

3.1.4 Expression of *pomc* mRNA

Temporal expression of *pomc* in the zebrafish embryonic development was investigated using RT-PCR. For these PCR the GSP5 and GSP6 (see appendix 6.2) were used and 700 bp cDNA fragment was amplified. Genomic DNA and dH_2O were additionally used as template for negative controls. Detection of the *pomc* mRNA from total RNA isolated from embryos at different developmental stages via RT-PCR revealed that the message is present in all studied stages. The pattern with strong expression in fertilized eggs, a weak expression in embryos at 6 hpf, and an increase thereafter demonstrates maternal expression of *pomc* and zygotic *pomc* RNA synthesis with a maximum in embryos at 28 h post-fertilisation (Fig. 11).

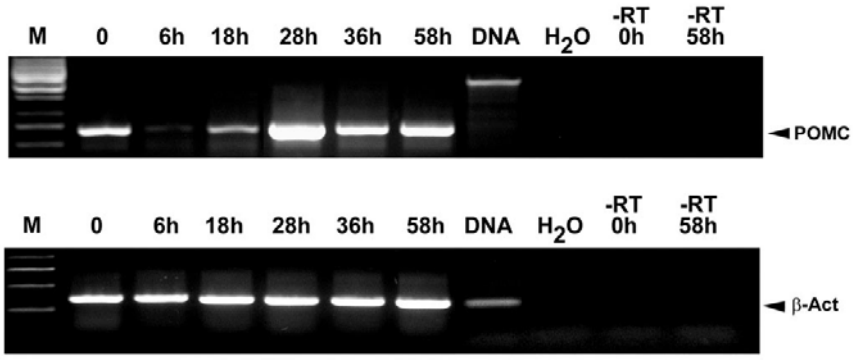

Fig. 11. Expression of zebrafish *pomc***.** Time course (in hours) of the *pomc* mRNA expression detected by amplification of a 700 bp cDNA fragment by RT-PCR. Genomic DNA (DNA) and H_2O were used as templates for controls. The numbers indicate hours after fertilisation. ß-actin (ß-Act) was used as RNA loading control (for primers see appendix 6.2). "–RT" denotes PCR without reverse transcription.

3.2 Development of zebrafish interrenal organ

In teleosts, the interrenal organ is composed of two cell populations with different embryonic orgins (Grassi Milano et al., 1997; Nandi, 1962): the steroidogenic and the chromaffin cell populations. Here, development of both cell populations in zebrafish as well as their co-development were analysed.

3.2.1 Development of the steroidogenic component of the interrenal organ and temporal expression of *ff1b*, *star*, *cyp11a1*, *mc2r,* and *3ß-hsd*

ff1b has been reported as the zebrafish homologue of the mammalian steroidogenic factor 1 (SF-1) as it is required for steroidogenic interrenal development and expression of steroidogenic genes (Chai et al., 2003; Hsu et al., 2003). To investigate wild-type zebrafish interrenal development, double *in situ* hybridizations (ISH) of *ff1b*, and of the steroidogenic acute regulatory protein (*star)* were performed at different time points from 18 hours post fertilisation (hpf) to 7 days post fertilisation (dpf) (Fig. 12).

Zebrafish interrenal primordium becomes detectable at 22 hpf as bilateral clusters of *ff1b* expressing cells (Fig. 12A), ventral to the 3rd somite. These two cell domains then fuse to a single cell cluster, slightly asymmetrically located to the right of the notochord at around 24 hpf (Fig. 12C). Expression of the key steroidogenic genes *star* (Fig. 12, C and D) and *cyp11a1,* the gene encoding cytochrome p450 cholesterol side chain cleavage enzyme is initiated shortly thereafter (Tab. 2). From 4 dpf onwards, the steroidogenic component of the interrenal primordium develops further into a distinct bilobed organ lateral to the notochord with the right lobe in general larger than the left lobe (Fig. 12, G-M).

To analyze the effect on the protein level, enzymatic activity of 3-beta-hydroxysteroid dehydrogenase (3β-Hsd) was studied using a chromogenic reaction with etiocholan-3β-ol-17-one as 3β-Hsd substrate. ISH for *mc2r, cyp11a1* and enzymatic staining of 3ß-Hsd were also performed for the embryos at 22, 24, 28, 32, 36 hpf to assess temporally the initiation of expression of these genes. Tab. 2 shows the sequential onset of these gene expressions. Of note *star* and *cyp11a1* expression precede 3ß-Hsd activity and *mc2r* expression is detetable only after 32 hpf.

Tab. 2: Time of onset of zebrafish steroidogenic genes

Gene	*ff1b*	*cyp11a1*	*star*	*3ß-hsd**	*mc2r*
Onset of expression (hpf)	22	24	24	28	32

*assessed as chromogenic enzymatic activity

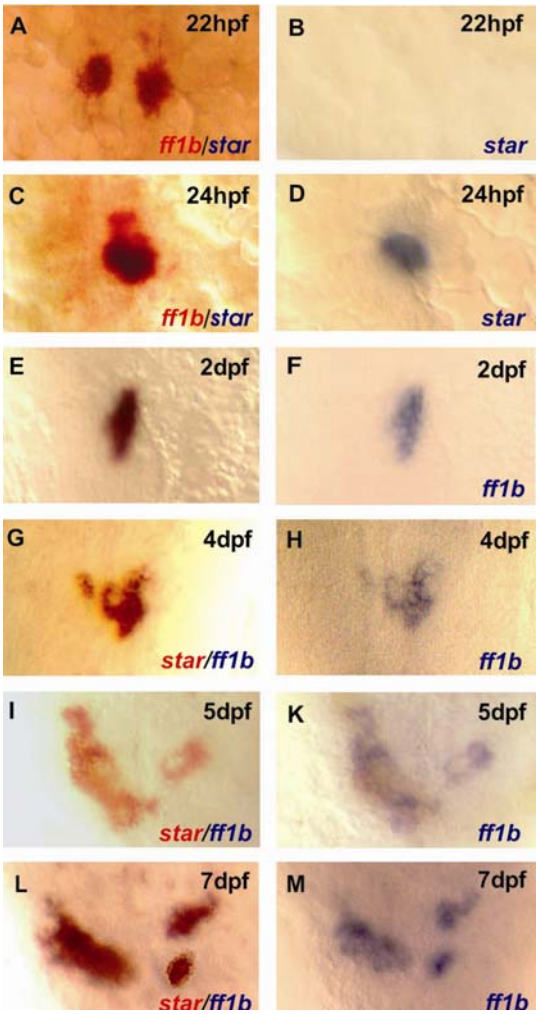

Fig. 12. Morphogenetic movement of the zebrafish interrenal primordium from 22 hpf to 7 dpf as assessed by two colour ISH. The probes (*ff1b* and *star)* are indicated in corresponding colours in the lower right corners. Age of the embryos is indicated in the upper right corners. A-H: dorsal view; I-M: ventral view. Anterior to the top. A, C: double ISH for *ff1b* (red) and *star* (blue). B, D: the blue *star* signal remains after the red ff1b signal in A, C, respectively is washed out. E, G, I, L: double ISH of *star* (red) and *ff1b* (blue). F, H, K, M: the blue *ff1b* signal remains after the red *star* signal in E, G, I, L, respectively was washed out. A: interrenal primordium is detected as bilateral groups of *ff1b* expressing cells at 22 hpf; B: no expression of *star* (but also of other interrenal markers) is detected at this stage. C: Fusion of interrenal primordium cells into one domain of *ff1b* expressing cells at 24 hpf, this cell population partly expresses other interrenal specific genes, e.g. *star* (D). E, F: At 2 dpf, the interrenal primordium remains as a single cell mass, in which *ff1b* and *star* expressing cells almost completely colocalize. G-M: colocalization of *ff1b* and *star*, further proliferation and development of the interrenal primordium into a bilobed interrenal organ from 4 dpf to 7 dpf.

From 2 dpf, all studied steroidogenic interrenal markers are completely colocalized in the interrenal primordium as also seen in 5 dpf embryos (Fig. 13).

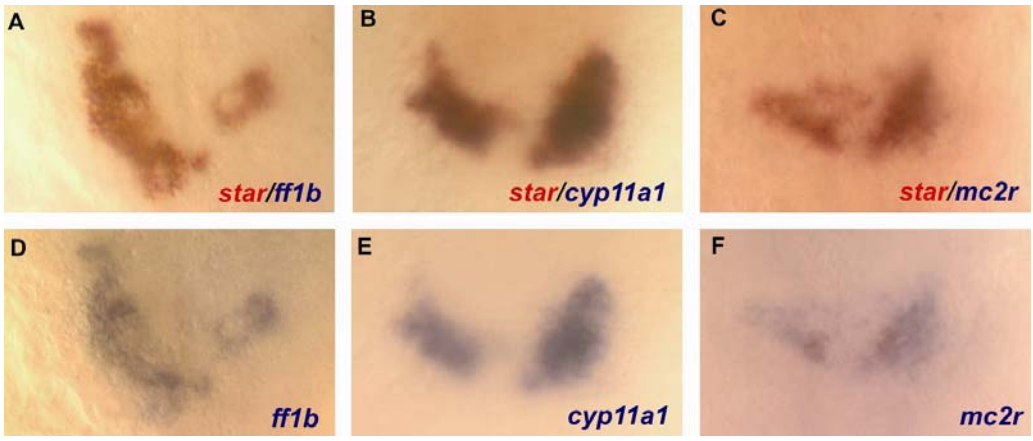

Fig. 13. Colocalization of *ff1b*, s*tar*, c*yp11a1* and *mc2r* in the interrenal primordium at 5dpf. Double ISH for *star* (red) and *ff1b* (A); *cyp11a1* (B); *mc2r* (C). A, D: ventral view; B, C, E, F: dorsal view. Anterior to the top. D, E, F: the blue signal of *ff1b, cyp11a1, mc2r* remains after red *star* signal in A, B, C, respectively was washed out.

3.2.2 Co-development of chromaffin and steroidogenic interrenal cells

In higher vertebrates, the adrenal cortex originates from the intermediate mesoderm, whereas the adrenal medulla is derived from neural crest cells (Else and Hammer, 2005; Hammer et al., 2005; Keegan and Hammer, 2002; Unsicker et al., 2005). To further investigate the organogenesis and morphogenetic movement of these two components of the interrenal organ in early developmental stages, transcripts of dopamine beta hydroxylase (*dβh*) as a marker for chromaffin cells and of *star* as a marker for steroidogenic cells were visualised by double ISH at different time points from 22 hpf-7 dpf in wild-type embryos (Fig. 14 and Fig. 15).

Dopamine β-hydroxylase (Dβh) converts dopamine to noradrenaline and is expressed in the chromaffin cells of teleosts (Grassi Milano et al., 1997; Reid et al., 1995). *dβh* expression was earliest detected in 22 hpf embryos as bilateral clusters of cells in the dorsal head region (Fig. 14, A and E), propably in sympathetic neurons (An et al., 2002).

No *dβh* expression was detected in the *star* expressing interrenal steroidogenic primordium until 36 hpf (Fig. 14, A-G).

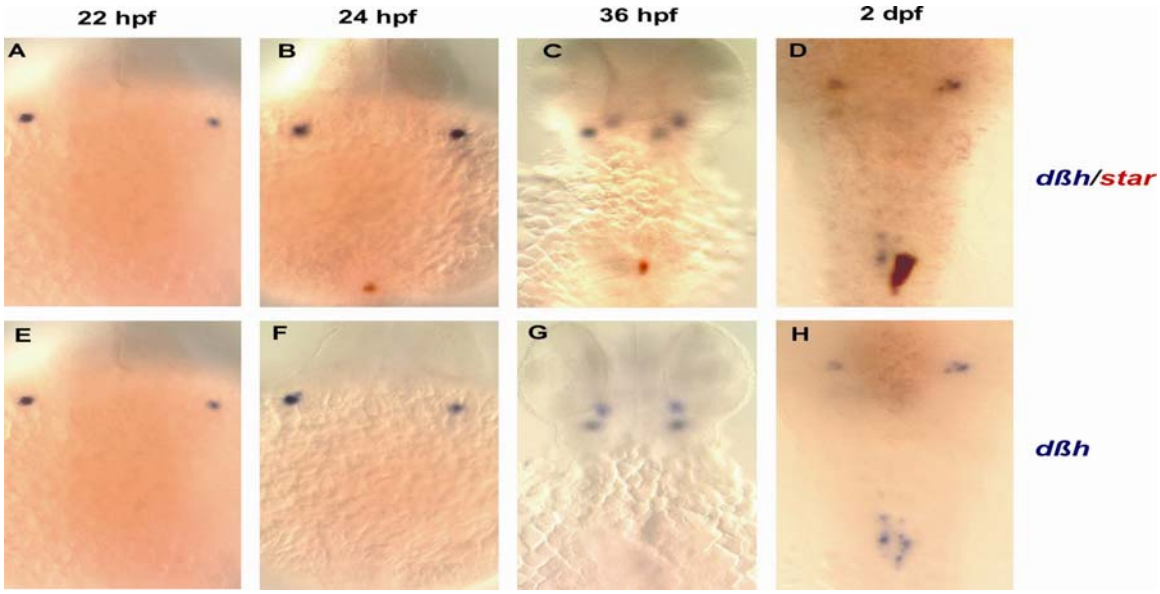

Fig. 14. *dβh* is not expressed in the zebrafish steroidogenic interrenal primordium until 36 hpf. A-D: double ISH for *dβh* (blue) and *star* (red). The age of embryo is indicated in the top of each column. E, F, G, H: the blue *dβh* signal remains after the red *star* signal in A, B, C, D, respectively was washed out. Dorsal view. Anterior to the top. *dβh* expression is ealiest detected in the steroidogenic interrenal primordium expressing *star* at 2 dpf in two domains lateral to the notochord (D, H).

Zebrafish chromaffin interrenal primordium converges to the region of the steroidogenic interrenal primordium at 2 dpf as bilateral domains of *dβh* expressing cells and overlap with the steroidogenic primordium in its right domain (Fig. 15, A and B). The expanding steroidogenic interrenal primordium then covers also the left *dβh* expressing cell domain (Fig. 15C). At 3 dpf, the two *dβh* expressing domains fuse to a single domain (Fig. 15, E and F) and develop further as a bilobed domain lateral to the notochord (Fig. 15, G-M) consistently in close contact to the steroidogenic cells. At 5-7 dpf the chromaffin cells are located closer to the trunk-midline than the steroidogenic cells and appear surrounded by the steroidogenic compartment of the interrenal organ (Fig. 15, I-M).

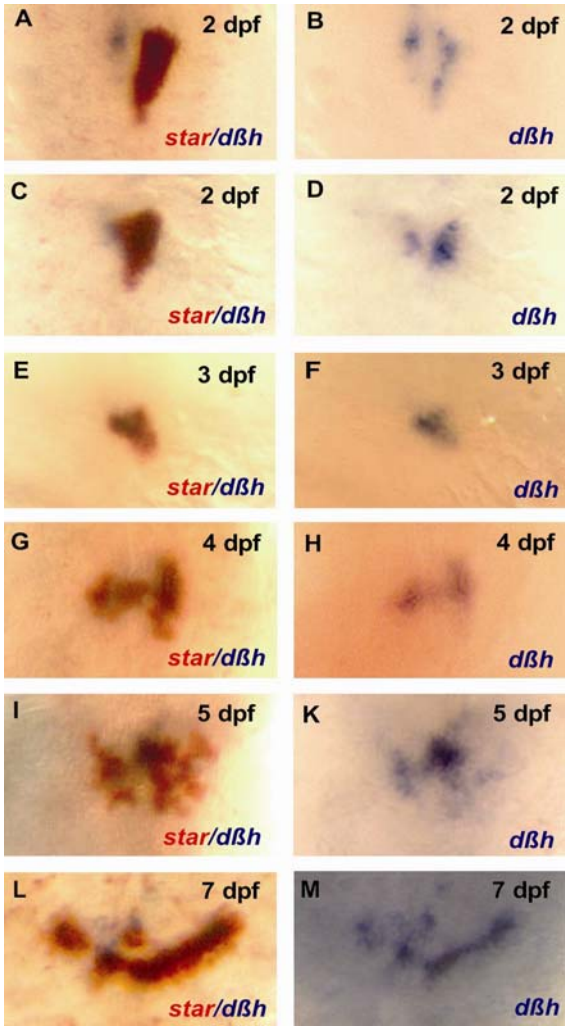

Fig. 15 Morphogenetic movement of steroidogenic cells in relation to that of chromaffin cells from 2 to 7 dpf. Double ISH of the steroidogenic marker *star* (red) and the chromaffin marker *dßh* (blue) was performed as indicated in corresponding colours in the lower right corner; dorsal view. Anterior to the top. Age of the respective embryos is indicated in the upper right corner. A, C, E, G, I, L: double ISH for *star* (red) and *dßh* (blue). B, D, F, H, K, M: the blue *dßh* persists after the red *star* signal in A, C, E, G, I, L, respectively, is washed out. A-D: Chromaffin cells migrate to the interrenal anlagen at 2 dpf as two clusters of *dßh*-expressing cells. Only the right cluster overlaps with the steroidogenic cells (A, C). C, D: the steroidogenic primordium expands towards the left domain of the chromaffin primodium. E, F: the steroidogenic cells completely cover the fused chromaffin cells at 3 dpf. G-M: Interrenal and chromaffin cells further proliferate and develop into a bilobed organ located on both sides of the notochord, with the right lobe being larger than the left lobe, as seen in the embryos from 4-7 dpf. During this development chromaffin cells appear enveloped by steroidogenic cells.

3.3 Pituitary-interrenal interactions in zebrafish interrenal organ development

3.3.1 Mutants that lack pituitary cells including corticotrophs show normal early interrenal development

To study the role of the pituitary in regulating early development of zebrafish interrenal organ, we analyzed the expression of zebrafish steroidogenic genes *cyp11a1, mc2r, star,* and of chromaffin marker gene *dβh* in *aal/eya1* and *lia/fgf3* mutants. *aal/eya1* and *lia/fgf3* are zebrafish mutants from a zebrafish ENU mutagenesis screen for mutations affecting adenohypophysis development (Herzog et al., 2004b). Of the different pituitary cell types (somatotrophs, lactotrophs, thyrotrophs, melanotrophs, corticotrophs, and gonadotrophs), *aal/eya1* mutants lack melanotrophs, corticotrophs, and gonadotrophs (Nica et al., 2004), whereas *lia/fgf3* mutants lack all pituitary cell types (Herzog et al., 2004a; Herzog et al., 2004b). Thus both mutants lack corticotrophs and therefore, expression of pituitary *pomc* is missing (Fig. 16, B and D).

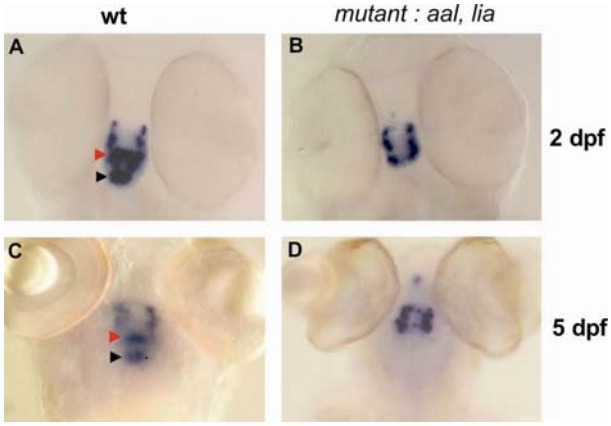

Fig. 16. *pomc* expression of *aal/eya1* and *lia/fgf3* mutants (*aal, lia*) compared to that of their wild-type (wt) siblings at 2 dpf (A, B) and 5 dpf (C, D). ISH of *pomc*. Dorsal view. Anterior to the top. The mutants have no pituitary *pomc* transcripts at both 2 dpf (B) and 5 dpf (D), whereas their wild-type siblings (A, C) exhibit an anterior (indicated by red arrowhead) and a posterior domain (indicated by black arrowhead) of pituitary *pomc* transcripts. Hypothalamic *pomc* transcripts that form two longitudinal stripes just above *pomc* expressing anterior pituitary as seen in wild-type embryos (A, C) are, however, still present in the mutants (B, D).

ISHs were therefore performed simultaneously for *pomc* and one or two interrenal markers to identify the mutants and analyse their interrenal phenotypes.

Fig. 17 shows interrenal phenotypes of *aal/eya1* and *lia/fgf3* mutants compared to their respective wild-type sibling at 2 dpf with respect to expression of steroidogenic genes *cyp11a1, mc2r, star,* and the chromaffin gene *dβh.*

In both mutants the transcripts of all studied interrenal genes are detectable and the expression pattern of all these genes remains unchanged until 2 dpf compared to wild-type embryos (Fig. 17). Similarly, at 2 dpf, no changes were observed in the morphogenetic movement of steroidogenic and chromaffin cells compared to those of wild-type embryos (Fig. 17, I-M). Enzyme activity of 3ß-Hsd was not analyzed in pituitary mutants for methodological reasons, as at 2dpf phenotypic differentiation between wild-type and mutant embryos is not possible without concomitant analysis of *pomc* expression

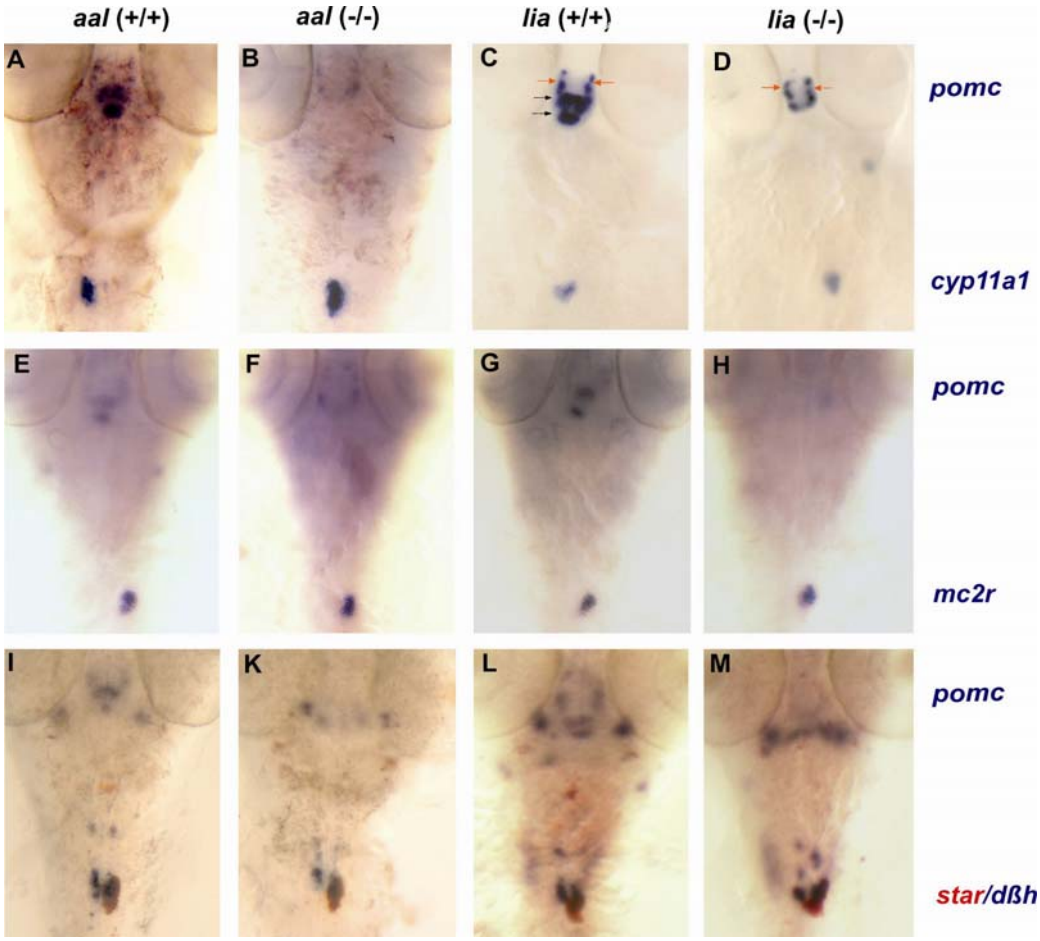

Fig. 17. Normal steroidogenic and chromaffin interrenal development of *aal/eya1* and *lia/fgf3* mutants at 2 dpf. A-D: ISH for *pomc* (upper signals) and *cyp11a1* (lower signals); E-H: ISH for *pomc* (upper signals) and *mc2r* (lower signals); I-M: ISH for *pomc* (upper signals) and *star/dßh* (lower signals). The markers are given in corresponding colours on the right side of the respective row; dorsal view. Anterior to the top. The first column shows wild-type sibling embryos of *aal/eya1* mutants (aal (+/+)); the second column shows *aal/eya1* mutant embryos (aal (-/-)); the third column shows wild-type sibling embryos of *lia/fgf3* mutants (lia (+/+)), and the fourth column shows *lia/fgf3* mutant embryos (lia (-/-)). The mutants have no pituitary *pomc* transcripts, as seen in B, D, F, H, K, M, whereas their wild-type siblings exhibit an anterior and a posterior domain of pituitary *pomc* transcripts, as seen in A, C, E, G, I, L, respectively. Hypothalamic *pomc* transcripts are, however, still present in the mutants, as is seen most clearly in D. No significant differences are seen in the mRNA expression pattern of steroidogenic genes (*cyp11a1, mc2r, star*) and *dßh* between mutants and their wild-type siblings (B, F, K vs A, E, I, respectively for *aal/eya1* mutants; D, H, M vs C, G, L, respectively for *lia/fgf3* mutants).

3.3.2 Mutants without pituitary cells (including corticotrophs) exhibit impaired interrenal steroidogenic function at day 5 post fertilisation

Fig. 19 demonstrates expression of the studied interrenal steroidogenic genes of *aal/eya1* and *lia/fgf3* mutants and their wild-type siblings at 5dpf. Photos were taken at higher magnification and focused only on the interrenal primordium. Significant reduction of mRNA expression of *cyp11a1, star, mc2r* as assessed by ISH was evident in both mutants at 5 dpf (Fig. 19, A-M) indicating that at this stage these genes are partly controlled by the pituitary gland. Using densitometric analysis both area and density of *cyp11a1* signal was significantly reduced in 5 dpf *aal/eya1* mutants (n=10) (density 45.8 ± 7.6% and area 45.5 ± 6.8%, $p<0.01$, x ± SEM, compared to wild-type embryos (n = 17) (Fig. 18). To analyse the effect on the protein level, activity of 3ß-Hsd was also studied. Both intensity and area of the staining for 3ß-Hsd activity was weaker in the mutants compared to wild-type (Fig. 19, N-Q) indicating a role of the pituitary at 5 dpf not only at the transcriptional level but also at the level of enzyme function.

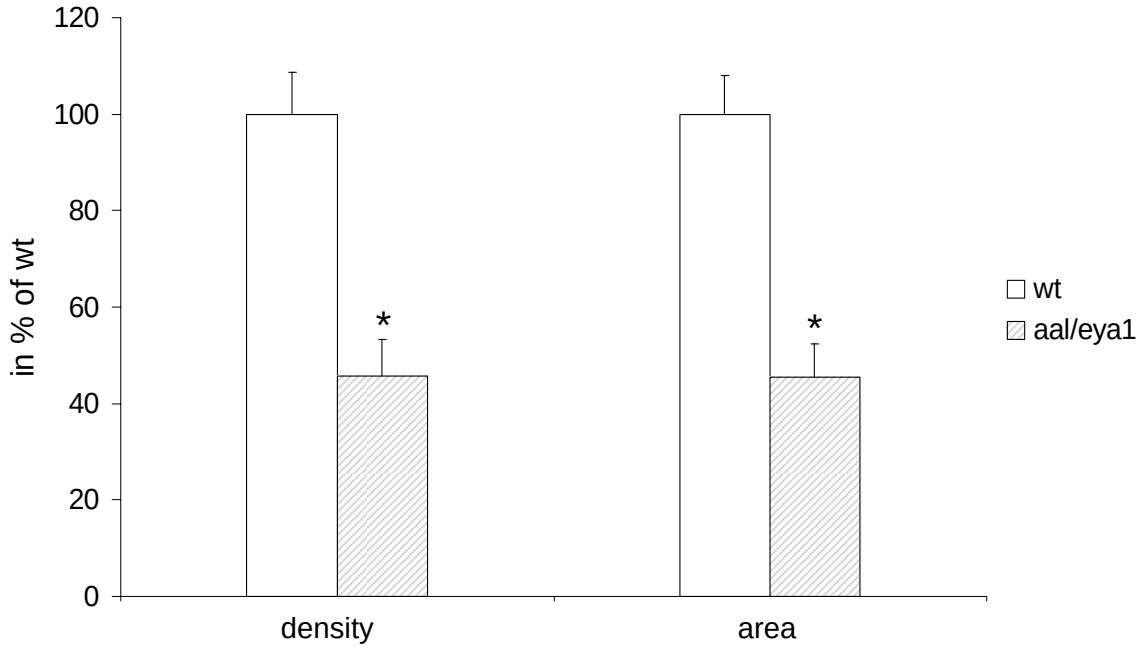

Fig. 18. density and area of the *cyp11a1* transcript signals assessed by ISH in *aal/eya1* mutants (aal/eya1) (n=10) compared to wild-type embryos (wt) (n=17), * $p<0.01$, x ± SEM.

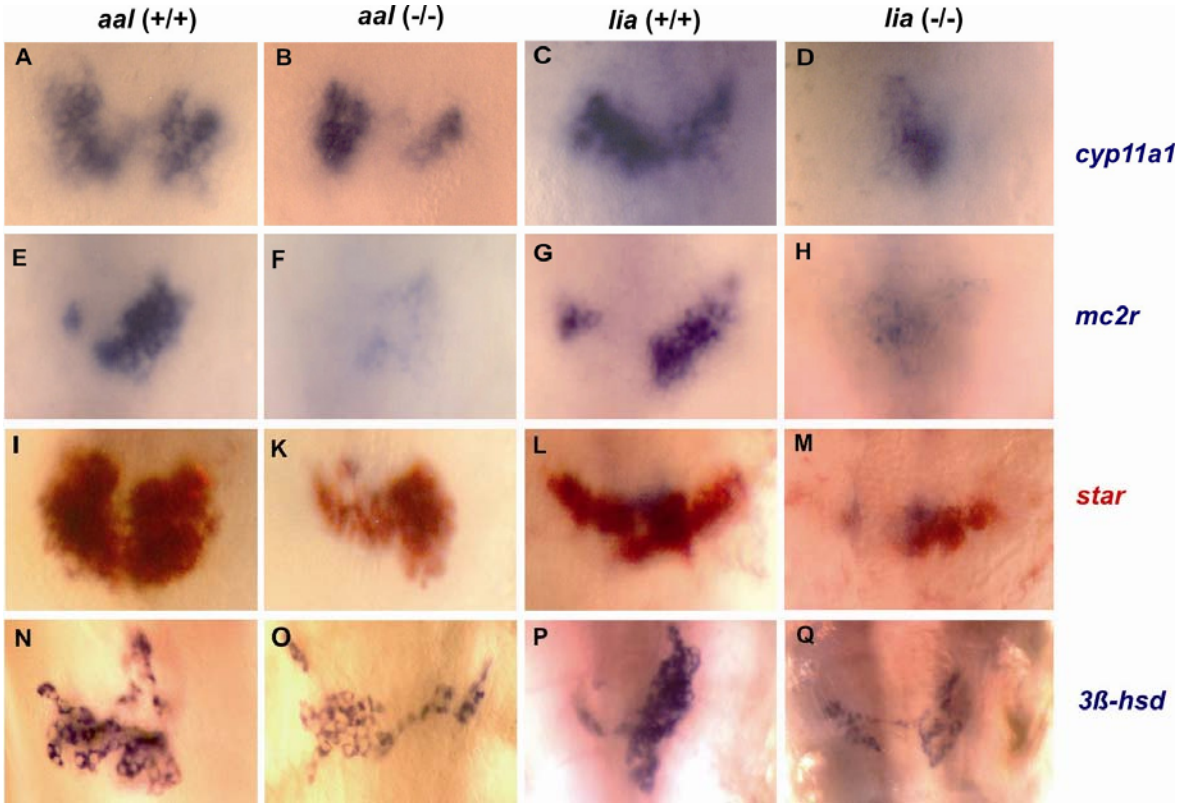

Fig. 19. Expression of steroidogenic genes in the *aal/eya1* and *lia/fgf3* mutants at 5 dpf. Single ISH for *cyp11a1, mc2r, star* and histochemical staining for 3ß-Hsd of *aal/eya1 (aal(-/-))* (B, F, K, O) and *lia/fgf3 (lia(-/-))* (D, H, M, Q) mutants and their corresponding wild-type siblings (A, E, I, N for *aal/eya1* siblings *(aal(+/+))* and C, G, L, P for *lia/fgf3* siblings *(lia(+/+))*. A-D, N, O: ventral view; E-M, P, Q: dorsal view. Anterior to the top. In both mutants, expression of steroidogenic genes *(cyp11a1, mc2r, star)* is significantly reduced at both the transcriptional level (B, F, K and D, H, M), compared to wild-type siblings (A, E, I and C, G, L, respectively) and functional level (O, Q), compared to wild-type siblings (N, P, respectively). In the mutants, the reduction of *mc2r* expression is more profound (F, H) than that of other interrenal markers (B, D, K, M).

3.3.3 Only pituitary corticotrophs are required to maintain normal interrenal development

To find out whether the effects in the 5 dpf *aal/eya1* and *lia/fgf3* mutants were caused by lack of corticotrophs and consequently by lack of pituitary Pomc or also by the lack of other pituitary cell types, the interrenal development in *pit1* mutants was studied at 5 dpf. Different from *aal/eya1* and *lia/fgf3* mutants, *pit1* mutants lack all pituitary cell types with the exception of corticotrophs and melanotrophs. *pit1*-null mutants were identified by ISH for *pit1*, as they exhibit no *pit1* transcripts (Nica et al., 2004). Fig. 20 shows expression of *pomc* and *pit1* in *pit1* mutant compared to its wild-type sibling as assessed by double ISH.

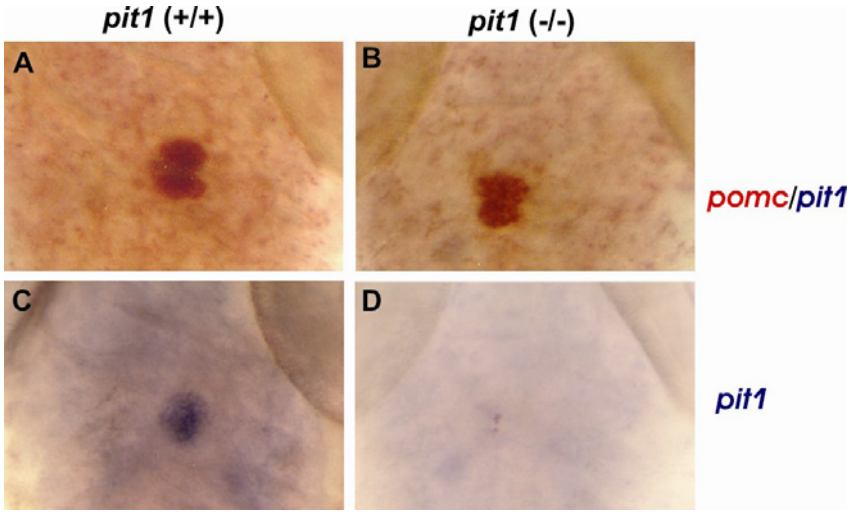

Fig. 20. *pit1* and *pomc* expression of *pit1* mutants analysed at 5 dpf. Double ISH of *pomc* (red) and *pit1* (blue) as indicated with corresponding colours on the right side for *pit1* wild-type sibling *(pit1(+/+))* (A) and *pit1* mutant *(pit1(-/-))* (B). C, D: remain of *pit1* signal after the red *pomc* signal in A, B respectively was washed out. Dorsal view. Anterior to the top. *pit1* mutant exhibits pituitary *pomc* (B) as wild-type (A) but has no *pit1* transcripts (D vs C).

Since *pit1* and interrenal gene expression are spatially separated, ISH was performed simultaneously for *pit1* and one or two steroidogenic interrenal markers to identify the mutants and analyse their interrenal phenotypes.

pit1 mutants showed normal interrenal development at 5 dpf with normal expression of all analyzed interrenal genes (Fig. 21) indicating that *pomc* expressing pituitary cells are fully sufficient to maintain normal development of the steroidogenic interrenal compartment.

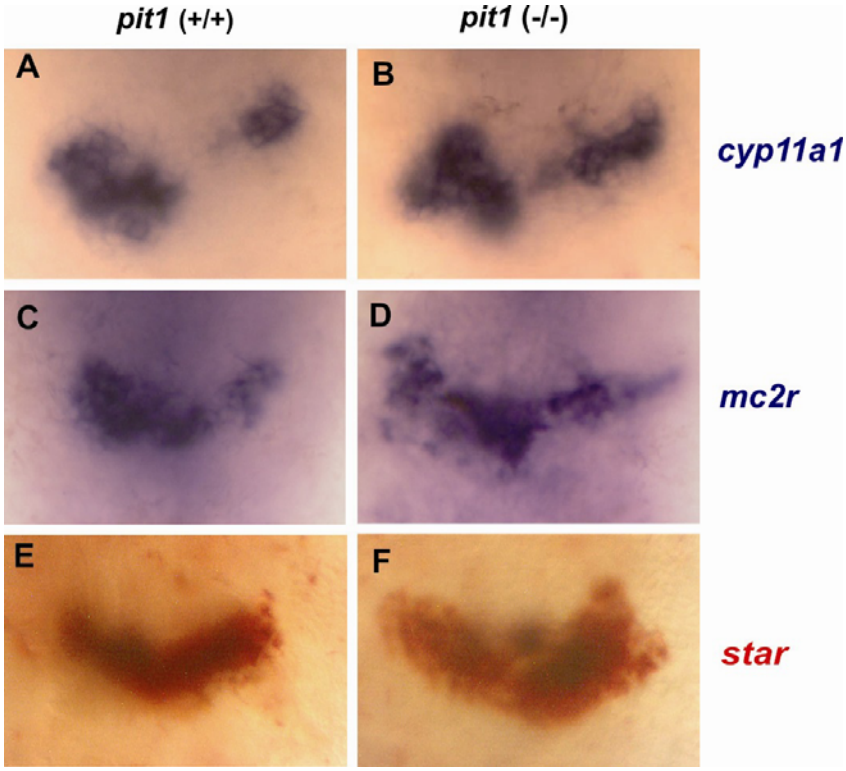

Fig. 21. Normal interrenal development of the *pit1* mutants at 5 dpf. Left column shows *pit1* wild-type sibling embryos (*pit1(+/+)*); right column shows *pit1* mutant embryos (*pit1(-/-)).* Single ISH of *cyp11a1* (A-B), *mc2r* (C-D), and *star* (E-F). A-D: ventral view; E-F: dorsal view. Anterior to the top.

3.3.4 *mc2r* receptor knockdown leads to a similar phenotype as *aal/eya1* and *lia/fgf3* mutants

To further clarify the role of Pomc peptides, morpholino technique (Nasevicius and Ekker, 2000) was used to knockdown the mc2 receptor (*mc2r* or *acth-r*) and the development of the steroidogenic and chromaffin compartments in the knockdown embryos was analysed. Morpholinos have been shown to efficiently block gene function in zebrafish embryos. They bind to mRNA and prevent translation of the target gene (Corey and Abrams, 2001; Summerton and Weller, 1997). In this study antisense *mc2r* morpholino was designed against the 5' UTR of the *mc2r* cDNA. In addition, a five-nucleotide-mismatch *mc2r* morpholino was also used to confirm the specificity of the morpholino.

3.3.4.1 Efficacy of *mc2r* antisense morpholino

Efficacy of *mc2r* antisense morpholino was confirmed by GFP based experiments (see 2.2.19). *mc2r* antisense morpholino blocked GFP translation of *mc2r*-GFP constructs. As expected, no GFP expression was observed in the 20 embryos injected with antisense *mc2r* morpholino in combination with the *mc2r*-GFP RNA, whereas strong GFP expression was observed in 19 of 21 embryos injected with RNA alone (Fig. 22).

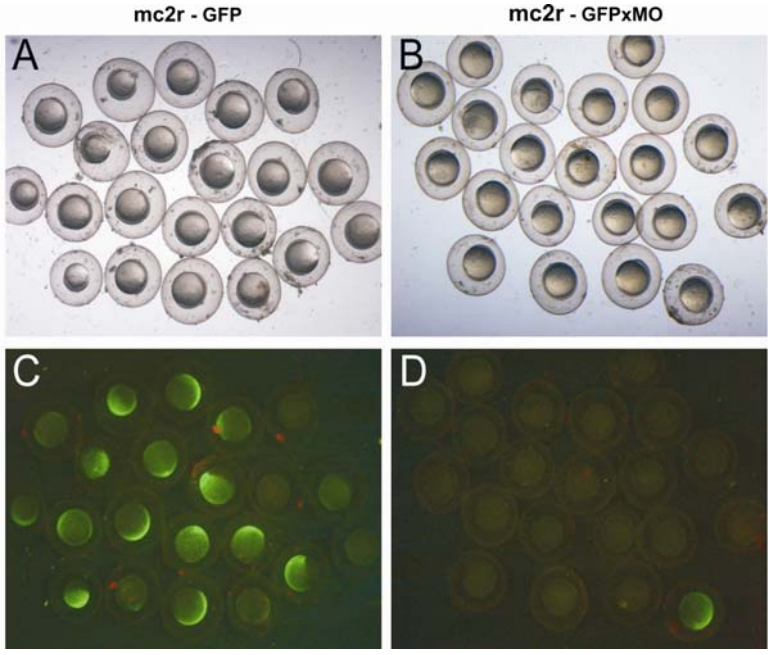

Fig. 22. Morpholino efficiency

Analysis of Morpholino efficiency. 50pg of RNA containing the 25nt target sequence for the *mc2r* Morpholino fused to a GFP reporter were injected alone (mc2r-GFP) or in combination with 8ng *mc2r* Morpholino (mc2r-GFPxMO) into zebrafish embryos at the one to two cell stage. C: Injection of the RNA alone resulted in strong GFP expression (in 19/21 embryos). D: GFP expression was significantly reduced in embryos coinjected with the *mc2r* Morpholino (no GFP expression in 20/20 embryos), embryo in the bottom right corner of D was injected with *mc2r* -GFP alone to serve as reference for fluorescence intensity. A, B: bright field; C, D: GFP fluorescence.

3.3.4.2 *mc2r* knockdown embryos show normal early interrenal development

In the *mc2r* antisense morphant embryos (asMO), similar results were obtained as in pituitary mutants *aal/eya1* and *lia/fgf3* lacking corticotrophs: expression of *cyp11a1*, *star*, *mc2r* and *dßh* remained completely unchanged until 2 dpf (Fig. 23), compared to those of wild-type and mismatch morphant embryos. At 2 dpf the transcripts of *pomc* in the *mc2r* knockdown embryos also remained unchanged (Fig. 23, arrow heads in A,B,C) suggesting no change in feedback signal at this stage of development.

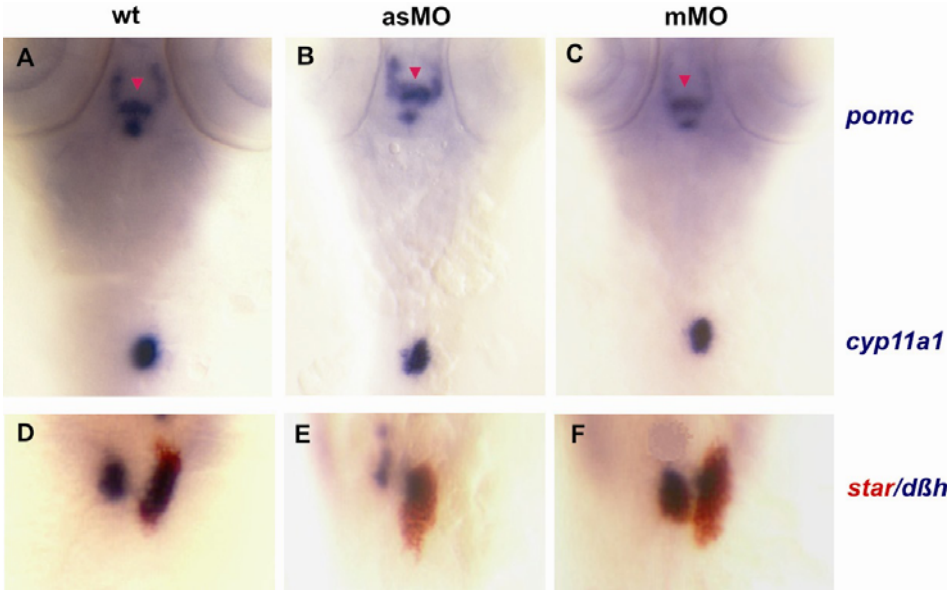

Fig. 23 Pituitary *pomc* , steroidogenic and chromaffin gene expression show no significant differences in the *mc2r* knockdown embryos (asMO) at 2 dpf, compared to wild-type (wt) and mismatch morphants (mMO). dorsal view. Anterior to the top. ISH for *pomc* and *cyp11a1* for the wt (A), antisense morphant (B), and mismatch morphant (C). Red arrow heads mark the anterior domains of *pomc* transcripts of the adenohypophysis. D-F: double ISH for *star* (red) and *dßh* (blue), photos were taken with higher magnification than A-C focussing on the interrenal region.

3.3.4.3 *mc2r* knockdown embryos exhibit impaired interrenal function at day 5 post fertilisation

At 5 dpf, significant reductions of mRNA expression levels of the interrenal marker genes *cyp11a1*, *star* were observed in *mc2r* antisense morphants (Fig. 24,B,E) compared to those of wild-type and *mc2r* mismatch morphants (Fig. 24, A,D,C,F) suggesting that lack of Acth activity alone has a similar effect as the lack of all pituitary Pomc derived peptides. Reduction of steroidogenic enzyme activity may cause glucocorticoid deficiency in the 5 dpf-*mc2r* knockdown larvae.

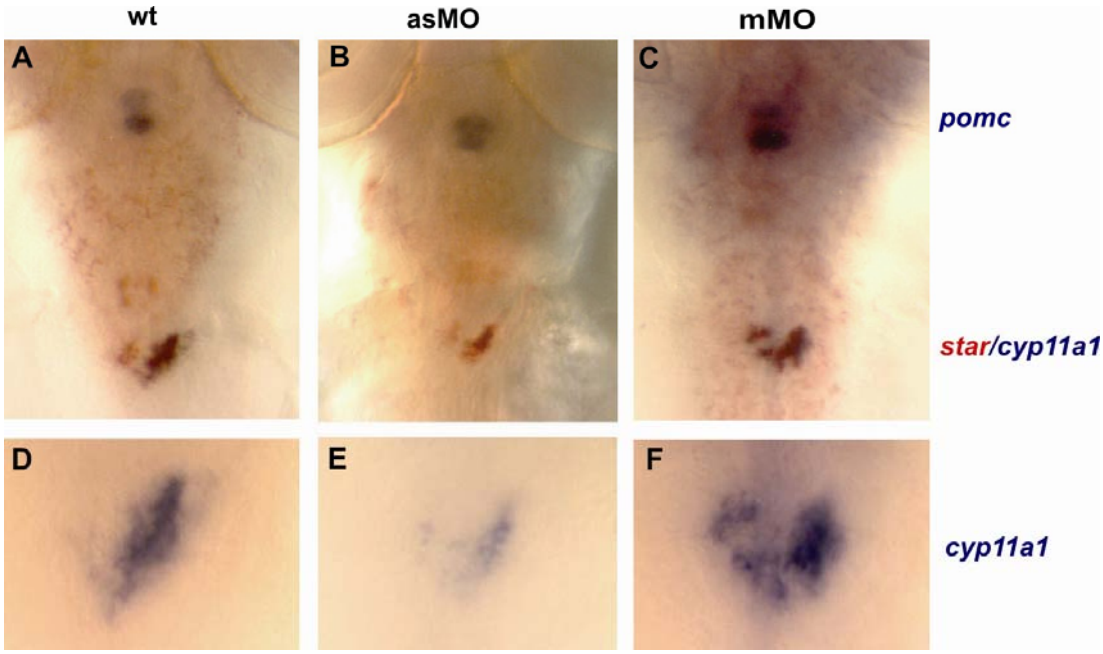

Fig. 24. Reduction of steroidogenic gene expression and increase of *pomc* expression in the embryos at 5 dpf caused by the knockdown of *mc2r* in the anterior domain of the adenohypophysis. Two colour ISH of *pomc* (blue) and *star* (red)/*cyp11a1*(blue) of the wild-type (wt) (A), *mc2r* antisense morphant (asMO) (B) and *mc2r*-mismatch morphant (mMO) (C) with the probes indicated in corresponding colours at the right side of the figure. Anterior to the top. D,E,F: larger magnification of *cyp11a1* signal in A,B,C, respectively, after the red *star* signal was washed out.

3.3.4.4 The knockdown of *mc2r* leads to an increased anterior pituitary *pomc* expression

To investigate evidence for feedback regulation we analysed *pomc* expression in *mc2r* knockdown larvae. *pomc* expression in *mc2r*-antisense morphants at 5 dpf was strongly increased in the anterior domain of the adenohypophysis (Fig. 25, B (red arrow head), D), compared to wild-type and mismatch morphants indicating that the anterior domain of *pomc* expressing cells is involved in glucocorticoid feedback regulation. In contrast, the posterior domain of pituitary *pomc* expressing cells (Fig. 25, B (black arrowhead), D) was not affected by *mc2r* knockdown.

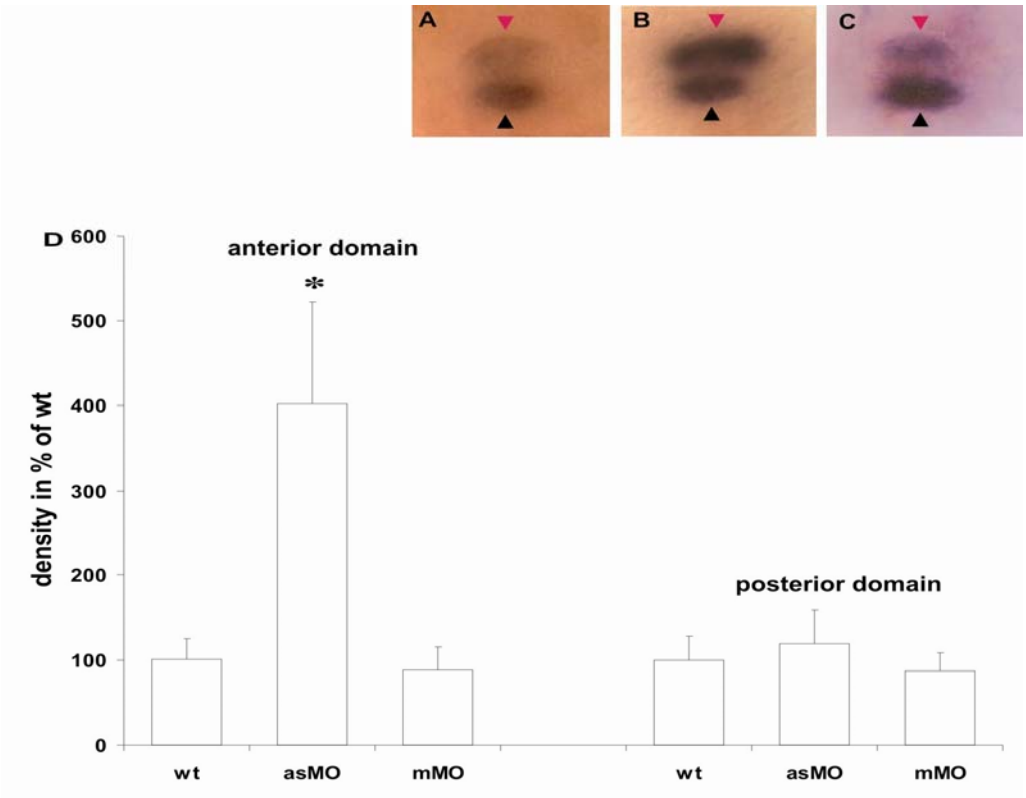

Fig. 25. Effect of *mc2r* knockdown on pituitary *pomc* expression at 5 dpf. A-C: *pomc* expression is increased in the anterior domain of adenohypophysis (marked by red arrow head in B), compared to that of wild-type (marked by red arrow head in A) and of mismatch morphant (marked by red arrow head in C),

whereas *pomc* expression in the posterior domain (marked by black arrows in A, B, C) remain the same. D. Densitometry of the *pomc* ISH signal (n=24-26 per group) is consistent with the observation from ISH in single embryo: *pomc* expression in the anterior domain of adenohypophysis is 4-fold increased compared with that of wild-type and mismatch morphants. *pomc* expression in the posterior domain remains unchanged; * $p<0.001$.

3.3.5 Sensitivity of the anterior pituitary to glucocorticoids precedes the response of the interrenal gland to pituitary Pomc peptides

The overexpression of endogenous *pomc* in the anterior domain of adenohypophysis in *mc2r* knockdown embryos suggested that only this domain of pituitary *pomc* expressing cells responds to glucocorticoid feedback. To further confirm this observation we treated wild-type embryos with dexamethasone (40µM) and analysed endogenous *pomc* and *cyp11a1* gene transcripts at different time points, varying from 2 to 5 dpf.

Dexamethasone led to a reduction of *pomc* transcripts in the anterior domain of corticotrophs as early as 2 dpf (red arrowhead in Fig. 26B). At 3 dpf, endogenous *pomc* expression in this domain was completely suppressed (red arrowhead in Fig. 26D) and also in 5 dpf embryos (red arrowhead in Fig. 26F). Again, the expression of *cyp11a1* was unaffected at 2 dpf (black arrowhead in Fig. 26B) but slightly down-regulated by dexamethasone at 3 dpf (black arrowhead in Fig. 26D). At 5 dpf dexamethasone treated embryos showed only weak expression of *cyp11a1* (black arrowhead in (Fig. 26F).

At the level of protein function, 3ß-Hsd activity was also significantly reduced in the 5 dpf dexamethasone treated embryos (Fig. 26H), compared with that of wild-type embryos (Fig. 26G). These results indicate that glucocorticoid feedback at the pituitary level precedes pituitary dependent steroidogenesis.

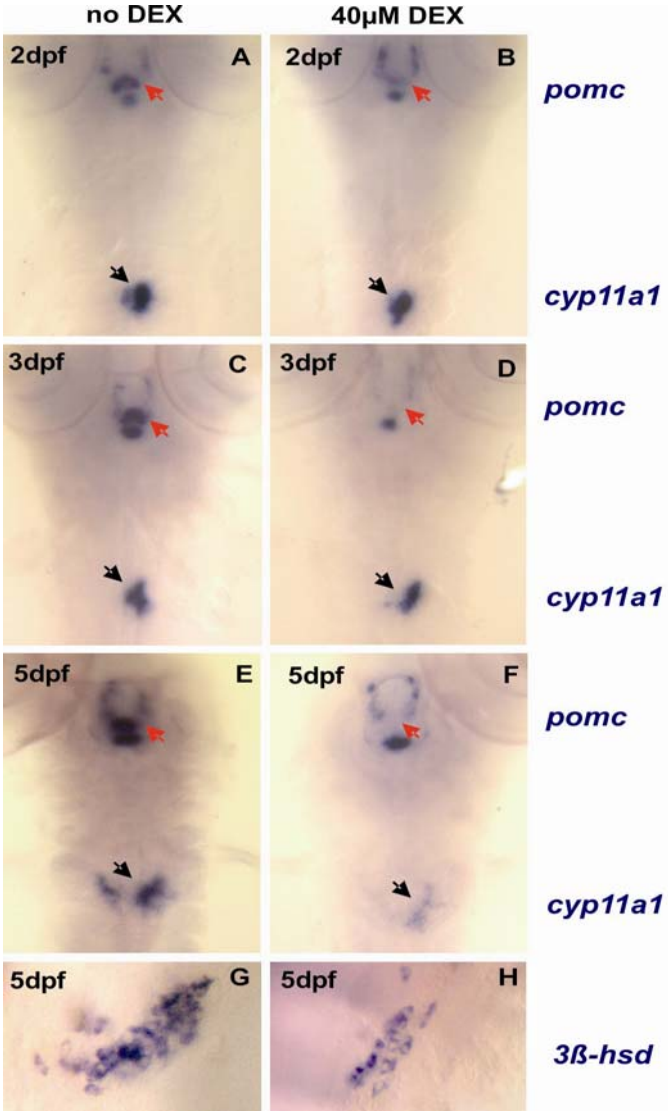

Fig. 26. *pomc* anterior and steroidogenic gene expression is down-regulated by dexamethasone. A-F: ISH of *pomc* (upper signal marked by red arrowhead) and *cyp11a1* (lower signal marked by black arrowhead); G,H: enzyme activity staining of 3ß-Hsd. Dorsal view. Anterior to the top. Age of embryos is indicated in the upper left corner and markers are indicated on the right side. The left column shows control embryos that were not treated with dexamethasone (no Dex); the right column shows dexamethasone treated (40µM Dex, starting at 1 dpf) embryos (40M DEX). Treatment with 40 µM dexamethasone down-regulates the expression of the *pomc* gene in the anterior domain of the adenohypophysis at 2 dpf (marked by red arrow head in B) vs controls (marked by red arrowhead in A) and suppresses *pomc* expression from 3dpf (marked by red arrowhead in D) vs control (marked by red arrowhead in C). The expression of *cyp11a1* is unaffected by dexamethasone treatment at 2dpf (black arrowhead in B, A) and is down-regulated from 3dpf treated embryos (black arrowhead in D, C) and mostly suppressed at 5 dpf (black arrowhead in E, F). Enzyme activity of 3ß-Hsd is also reduced but still detectable in dexamethasone treated embryos at 5dpf (H vs G). Downregulation of *pomc* expression in the anterior domain of the pituitary is already detectable at 2dpf and complete at 5dpf, whereas *pomc* expression in posterior pituitary domain remains unaffected.

3.4 Functional interaction between steroidogenic and chromaffin components in interrenal development

3.4.1 Lack of pituitary corticotrophs also leads to impaired expression of the chromaffin marker gene *dßh*

Studies in mammals and amphibians have shown increasing evidence that adenal cortex and adrenal medulla are functionally dependent on each other (DeLean et al 1984, Pratt et al 1985, Ehrhart-Bornstein et al 1995, Haidan et al 1998, Bornstein et al 1990, Bornstein et al. 2000 and Shepherd and Holzwarth 2001). To investigate this in zebrafish, expression of the chromaffin marker *dßh* was analysed by ISH in *aal/eyal*, *lia/fgf3* and in *pit1* mutants at 5 dpf. *aal/eyal* and *lia/fgf3* mutants not only exhibit a significant decrease of steroidogenic gene transcripts at 5dpf as described above (see 3.3.2), but also a remarkable reduction of *dßh* (Fig. 27, A-D). In the *pit1* mutant with a normal steroidogenic gene expression, also a normal *dßh* expression was demonstrated (Fig. 27, E and F). These results indicate that corticotrophs in pituitary mutants are both necessary and sufficient for the normal expression of *dßh*.

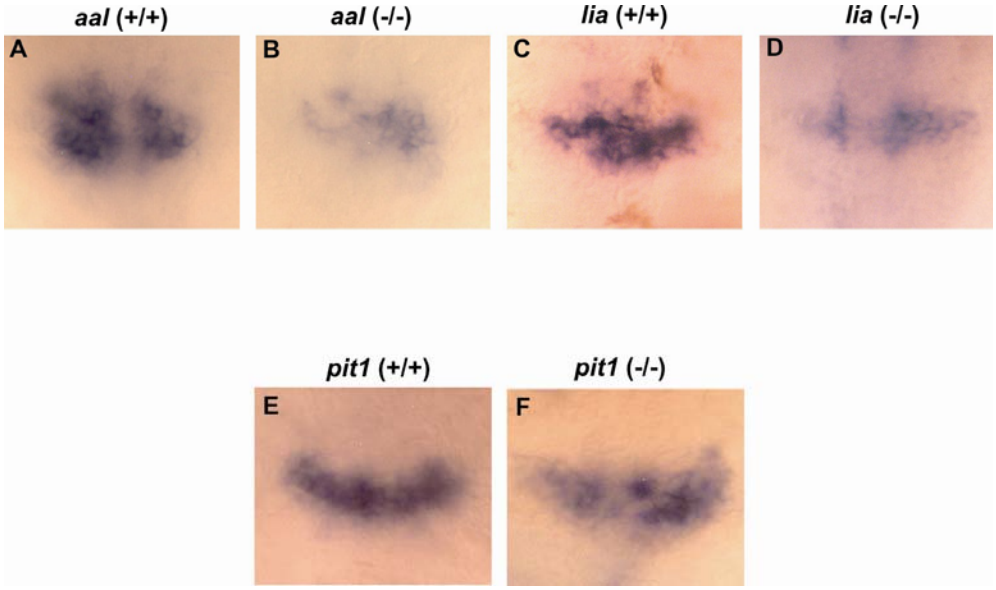

Fig. 27. Reduced *dßh* expression in *aal/eyal*, *lia/fgf3* mutants and normal *dßh* expression in *pit1* mutants at 5 dpf.

ISH for *dßh*. A,B: ventral view. C-F: dorsal view. Anterior to the top. Expression of zebrafish chromaffin marker gene *dßh* is also reduced in *aal/eya1* (B) and *lia/fgf3* (D) mutants, comprared to that of their corresponding silbling wild-type embryo (A for *aal/eyal* mutant; C for *lia/fgf3* mutant), wheares in *pit1* mutant expression of *dßh* remain unchanged (F), compared to wild-type (F).

3.4.2 *dßh* expression in *mc2r* knockdown embryos

Expression of *dßh* in *mc2r* knockdown embryos (asMO) was analysed via ISH, simultaneously with *pomc* to ensure the efficiency of the antisense morpholino. Photos were taken only from the embryos showing increased anterior pituitary *pomc*. The results are shown in Fig. 28. As in the *aal/eya1* and *lia/fgf3* mutants, chromaffin *dßh* expression in *mc2r* knockdown morphants (asMO) was decreased at 5 dpf, compared to wild-type and mismatch morphants (Fig. 28, E vs D and F, respectively).

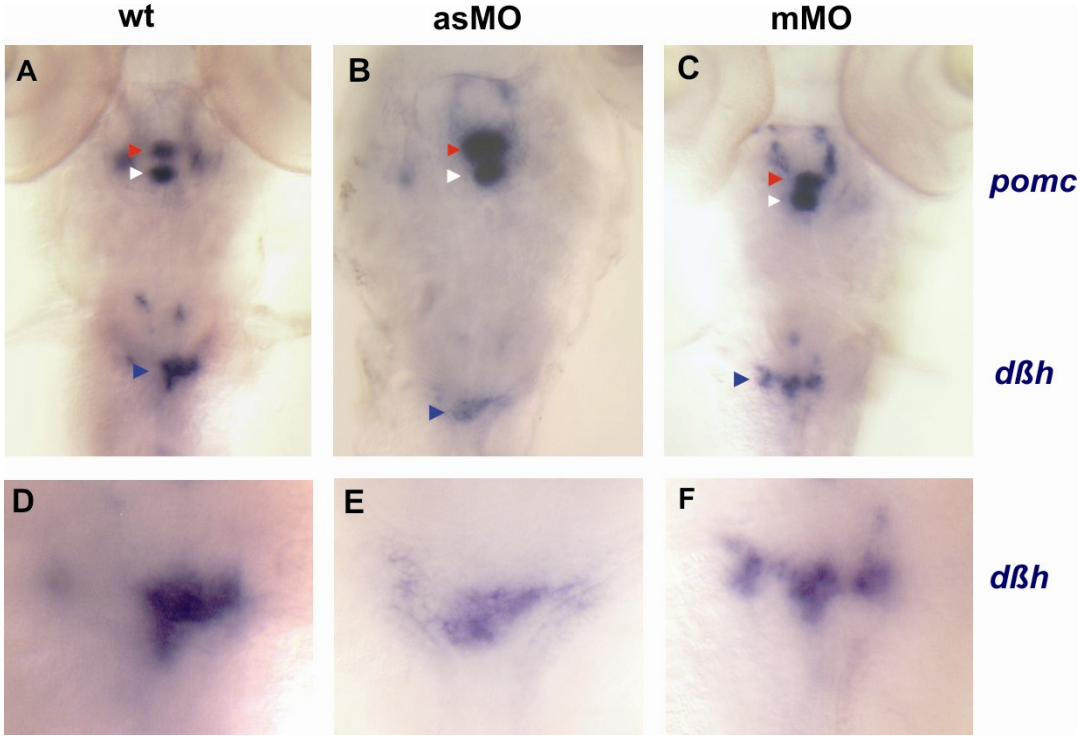

Fig. 28. *dßh* expression of the *mc2r* knockdown embryo (asMO), compared to that of wild-type (wt) embryo and mismatch morphant (mMO). ISH for the markers indicated on the right side of each row. Dorsal view. Anterior to the top. Anterior and posterior pituitary *pomc* expression are marked by red and white arrowheads, respectively in A, B, C. Blue arrowhead in A, B, C points at *dßh* expression in the interrenal

primordium which is demonstrated in higher magnification in D, E, F, respectively. In the *mc2r* knockdown embryos, increased of anterior pituitary *pomc* expression as well as decreased *dβh* expression is observed.

3.4.3 *dβh* expression in dexamethasone treated embryos

In the dexamethasone treated embryos at 5dpf, a dramatic decrease of steroidogenic *cyp11a1* gene expression and a complete suppression of anterior pituitary *pomc* expression were observed as described above (see 3.3.5). To investigate how dexamethasone affects expression of the chromaffin *dβh* gene, *dβh* transcripts were assessed by ISH in the wild-type with no DEX treatment and in the 40 µM DEX treated embryo, simultaneously with *pomc*. The results are given in Fig. 29.

While anterior pituitary *pomc* expression is completely suppressed by DEX (Fig. 29B), *dβh* expression in the DEX treated embryo seems slightly reduced at the area level but increased at the density level (Fig. 29D), compared to the wild-type embryos (Fig. 29C) indicating that the inhibitory effect of supressed *pomc* on *dβh* expression is rescued by exogenous glucocorticoids.

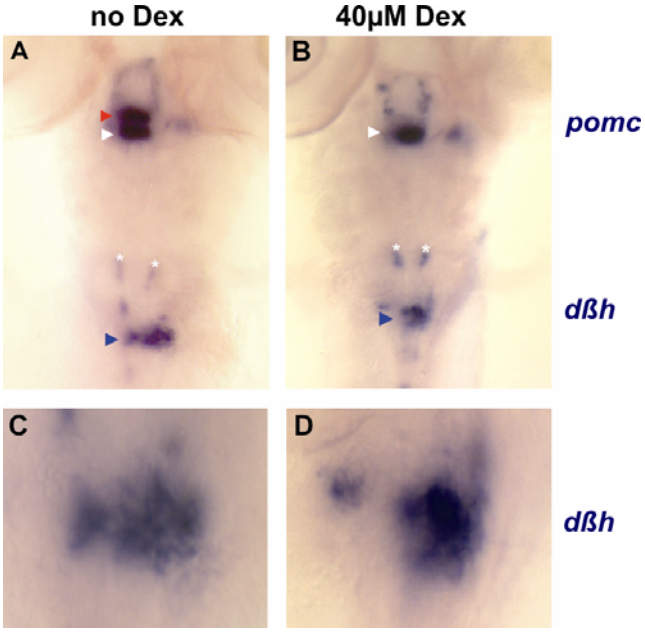

Fig. 29. *dβh* expression in dexamethasone treated embryos at 5 dpf. A,B: ISH for *pomc* and *dβh* as indicated on the right side with corresponding colours. Dorsal view. Anterior to the top. Red and white arrowheads

point at the anterior domains and the posterior domains of pituitary *pomc* expression, respectively. Blue arrowheads indicate *dβh* expression in the interrenal primordium. White asterisk marks *dβh* expression outside the interrenal primordium, probably representing the cervical sympathetic ganglion (An et al., 2002). C, D: larger magnification of the interrenal *dβh* expression indicated by blue arrowheads in A, B respectively. While the area of of *dβh* expression seems to be slightly decreased in the DEX treated embryos, the intensity of the expression is somewhat stronger.

3.5 Zebrafish "*pomc-like*" gene plays no role in regulating interrenal steroidogenesis and expression of interrenal genes

Due to further completion of the zebrafish genomic sequence, recently a homologous sequence to the zebrafish *pomc* gene *("pomc-like")* has been reported to be present in the zebrafish genome (de Souza et al., 2005; Gonzalez-Nunez et al., 2003). Characterisation of this Pomc homologue showed that it contains the consensus sequences for Acth, γ-Lph, and β-End but only β-End and α-Msh are likely to be end products, as the proteolytic cleavage sites needed for Acth and other peptides are mutated (Gonzalez-Nunez et al., 2003). However, the question was posed whether "*pomc-like*" may also play a role in regulating zebrafish interrenal development and interrenal gene expression or if the expression of interrenal genes in the *aal/eya1*, *lia/fgf3* and *mc2r* knockdown embryos at 5 dpf remains unaffected by this gene. Thus, the expression pattern of this *pomc* homologue was analysed in wild-type embryos, in *aal/eya1* mutants as well as in *mc2r* knockdown and dexamethasone treated embryos.

3.5.1 Expression pattern of "*pomc-like*" gene in wild-type and in *aal/eya1* mutant embryos

Partial cDNA of "*pomc-like"* was amplified by RT-PCR with total RNA from head of adult zebrafish. Primers were designed based on the sequence of "*pomc-like*" accession No. AL845420: forward primer: GTTCTGTCCGTCTTGGCTTT and reverse primer GGCAATGGACAGCAGTTCAC. PCR fragments were cloned into pCRII-TOPO vector and digoxigenin-labelled antisense RNA probe of "*pomc-like"* was synthesized as described in 2.2.13.1.

Double ISHs of *pomc* and "*pomc-like*" were performed for the wild-type embryos at 2 and 5 dpf and for the *aal/eya1* mutant at 5 dpf. Fig. 30 shows the expression pattern of these genes. "*pomc-like*" transcripts are detected in the brain region anterior to the pituitary expression region of the *pomc* gene at both 2 and 5 dpf. While the *pomc* gene was shown to be expressed in two cell domains in the pituitary and in two longitudinal stripes of cells in the hypothalamus anterior to the pituitary region, "*pomc-like*" expression is not found in the pituitary. However it was found to colocalize partially with *pomc* gene expression in the hypothalamus (Fig. 30).

In *aal/eya1* mutant at 5dpf, expression of "*pomc-like*" was also detectable (Fig. 30D) but in a reduced manner compared to that of wild-type embryo (Fig. 30, D and C).

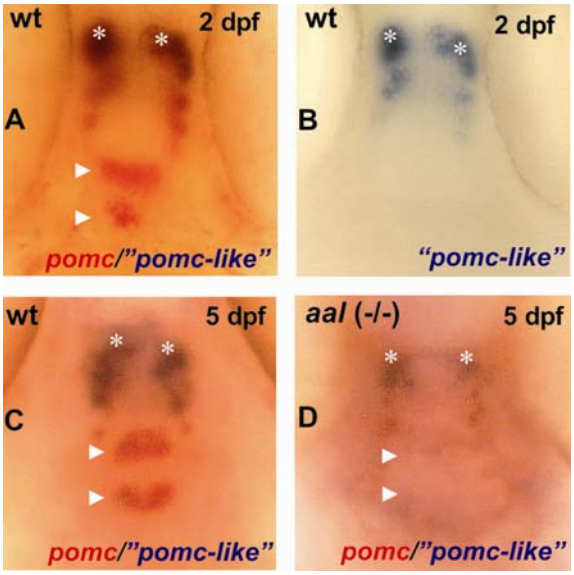

Fig. 30. "*pomc-like*" expression in relation to *pomc* expression in wild-type embryos (wt) at 2 and 5 dpf and in *aal/eya1* mutant (aal (-/-)) at 5 dpf. Age of the embryos is indicated at the upper right corners. A, C, D: Double ISH of *pomc* and "*pomc-like*" as indicated by corresponding colours in the lower right corners. B: the blue "*pomc-like*" signal remains after the red signal in A was washed out. Dorsal view. Anterior to the top. White arrowhead points at pituitary *pomc* expression. Asterisk marks "*pomc-like*" expression. At both 2 and 5 dpf, "*pomc-like*" is not expressed in the pituitary region as indicated by white arrowhead in A and C. "*pomc-like*" expression is located anterior to the pituitary *pomc* expression and partly overlaps with two longitudinal stripes of hypothalamus *pomc* expression in their posterior parts (A,B). In the *aal/eya1* mutant at 5 dpf, no pituitary *pomc* is available but "*pomc-like*" expression is detectable (white asterisks in D) with decreased level compared to wild-type (white asterisks in C).

3.5.2 *"pomc-like"* expression is unaffected by the knockdown of *mc2r* and by dexamethasone treatment

Fig. 31 shows expression of "*pomc-like*" gene in MC2R knockdown (asMO) and in DEX treated (40µM DEX) embryos at 5 dpf. ISH of *pomc* (fluorescence labelled probe) and *cyp11a1* (digoxigenin labelled probe) was performed in the embryos at 5 dpf.

Although in *mc2r* knockdown embryos and DEX treated embryos decreased expression of the steroidogenic gene *cyp11a1* and increased (in *mc2r* knockdown embryos) or suppressed (DEX treated embryos) anterior pituitary *pomc* were observed, expression of "*pomc-like"* was unaffected by DEX or *mc2r* knockdown. These results indicate that "*pomc-like***"** has no role in regulating interrenal gene expression and function.

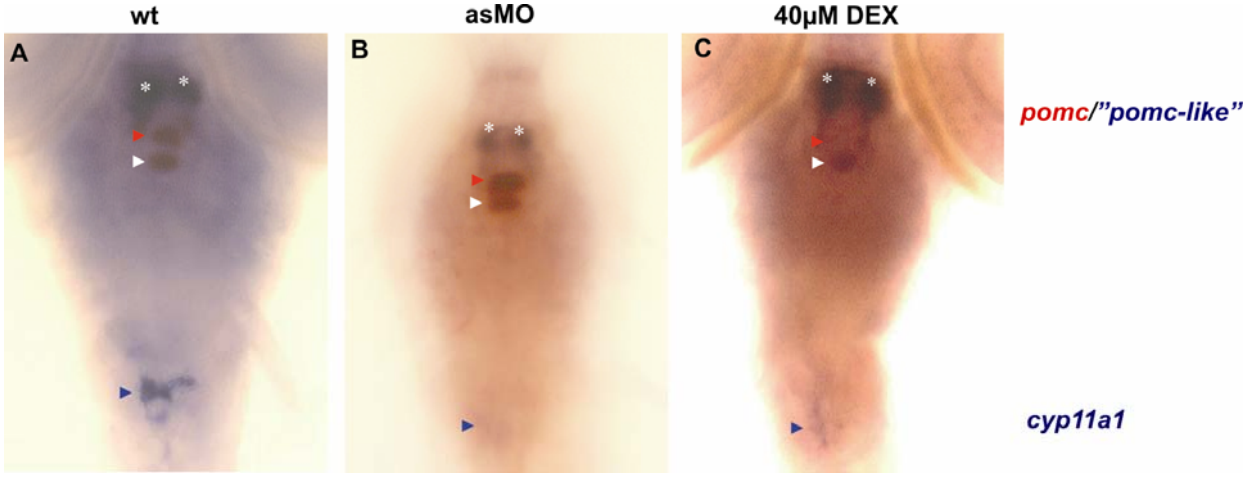

Fig. 31. "*pomc-like*"expression in *mc2r* knockdown (asMO) and dexamethasone treated (40µM DEX) embryos, compared to wild-type embryos (wt) at 5 dpf. ISH for *pomc*/"*pomc-like*" and *cyp11a1* as indicated with corresponding colours on the right. Upper red signals (pointed by red arrowheads for anterior domains and white arrowheads for posterior domains) are *pomc* transcripts, upper blue signals are "*pomc-like*" transcripts (marked by white asterisks). Lower blue signal indicates *cyp11a1* expression (blue arrowhead). Dorsal view, anterior to the top. In the *mc2r* knockdown (asMO) and DEX treated embryos, decreased expression of *cyp11a1* (marked by blue arrowhead in B, C, respectively vs A) is evident. Increased expression (asMO) or suppression (40µMDEX) of anterior pituitary *pomc* (pointed by red arrowheads in B, C, respectively) was observed. However, expression of *"pomc*-like*"* was unaffected by either *mc2r* knockdown (white asterisks in B) or by DEX (white asterisks in C).

4 Discussion

4.1 The zebrafish *pomc* gene and expression pattern of *pomc* homologue

In this study, the *pomc* gene of zebrafish was cloned and characterized. It shows high structural homology with higher vertebrate *POMC* genes. It consists of three exons and two short introns and has a similar overall structural organisation as in *homo sapiens*.

Proopiomelanocortin (POMC) is the preprohormone for ACTH and a number of peptide hormones involved in such diverse functions as stress adaptation, weight control, and perception of pain (Hadley and Haskell-Luevano, 1999). POMC has been found in all classes of vertebrates and also in invertebrates like leech and a marine bivalve molluscan (Salzet et al., 1997; Stefano et al., 1999). Most activities of POMC-derived hormonal peptides are mediated through melanocortin (MC) receptors (Strand, 1999). Recently, it has been shown that the known MC receptors from zebrafish share 70% overall amino acid identity with the respective human orthologues (Ringholm et al., 2002). Moreover, these receptors show pharmacological properties remarkably similar to their mammalian orthologues (Ringholm et al., 2002), indicating that zebrafish is a promising model in characterizing the physiology of POMC-derived hormones.

In tetrapods, POMC is composed of pro-γ-MSH, ACTH, and β-Lipotropin with each part containing one MSH domain (γ-MSH, α-MSH, and β-MSH, respectively). A similar structure has been found in lungfish and in primitive *actinopterygians* (Dores et al., 1999; Joss et al., 1990). In contrast, teleost Pomc lacks γ-Msh (Takahashi et al., 2001). Accordingly, the sequence of the zebrafish Pomc described here lacks a γ-Msh-like sequence, as also reported in *C. carpio* belonging to the same family (*cyprinidae) as* zebrafish. *Cyprinus carpio* has been shown to possess two closely related Pomc sequences (Arends et al., 1998a; Arends et al., 1998b). The Pomc amino acid sequence of *D. rerio* has a high degree of sequence similarity with the two carp Pomcs, as expected from the close relationship of these species. Moreover, phylogenetic analysis (Hansen et al., 2003) showed that the zebrafish Pomc sequences reflect the expected pattern of species evolution.

Recently, Gonzalez-Nunez et al. have described a homologous sequence to zebrafish *pomc* called "*pomc-like*" or *pomc ß* in the zebrafish genome (Gonzalez-Nunez et al., 2003). Sequence analysis revealed that the deduced polypeptide contains the consensus sequences for Pomc peptides, but only α-Msh and ß-End were considered as end products (Gonzalez-Nunez et al., 2003). Moreover, the ß-End appears in an inactive form, as it has no "opioid message" needed for the stimulation of opioid receptors (Gonzalez-Nunez et al., 2003). Thus, it is possible that this zebrafish "*pomc-like*" sequence represents part of a *pomc* duplicate, which might have appeared as a result of a whole-genome duplication that has taken place in the *cyprinidae* family or even in all teleosts (de Souza et al., 2005; Gonzalez-Nunez et al., 2003).

The function of this gene has not yet been elucidated. The investigations presented in this work demonstrate that "*pomc-like*" is expressed in the hypothalamus in a region anterior to hypothalamic *pomc* with some overlap between *pomc* and "*pomc-like*" expressing cells. Different from pituitary *pomc* expression, "*pomc-like*" gene expression is not affected by glucocorticoids and plays no role in interrenal controls (see 3.5.2). Thus these findings indicate that "*pomc-like*" gene is not involved in endocrine activity but rather represents a local hypothalamic activity. Intriguingly in *aal/eya1* mutants "*pomc-like*" expression is reduced indicating a physiological role of *eya1* for "*pomc-like*" expression, whereas hypothalamic *pomc* expression remained unaffected. As α-Msh is likely to be the major product of "Pomc-like", "*pomc-like*" is probably involved in the hypothalamic control of body weight. Taken together, all the data gained here suggest a subfunctionalization of a *pomc* duplicate following ancient duplication of *pomc* gene, as has been reported in other teleosts (de Souza et al., 2005; Gonzalez-Nunez et al., 2003).

Structural analysis of zebrafish Pomc obtained in this study is consistent with data reported by Liu et al., 2003a. Comparison of the amino acid sequences of man and zebrafish reveals strong conservation for α-MSH, ACTH, β-MSH, and β-endorphin. Although the N-terminus of Pomc (pro-γ-Msh) in the zebrafish lacks a γ-Msh sequence, there is high homology in the N-terminal 44 amino acids, suggesting an important physiological role of this peptide. This domain of Pomc contains two disulphide bridges at its extreme N-terminal end, inducing a hairpin tertiary structure that is essential for targeting POMC to the regulated secretory compartment of pituitary corticotrophs (Loh et al., 2002). The

specific sorting motif, consisting of two acidic and two hydrophobic residues (Asp10, Leu11, Glu14, and Leu18), is necessary for targeting Pomc to the regulated secretory pathway and is conserved in zebrafish N-Pomc (Loh et al., 2002). In mammals, pro-γ-MSH has been shown to stimulate adrenal growth (Estivariz et al., 1982) and to increase ^{3}H-thymidine incorporation into lactotrophs (Bert et al., 1999; Denef et al., 2001). These mitogenic activities seem to be unrelated to the γ-MSH component and likely reside in the highly conserved N-terminal domain of pro-γ-MSH (Bert et al., 1999; Denef et al., 2001). Accordingly, Fassnacht et al. have found that 1-28-N-POMC stimulates growth of adrenocortical cells *in vitro* (Fassnacht et al., 2003). This view is supported by studies in rainbow trout, which demonstrate hypertrophy of interrenal cells after administration of N-terminal peptides from trout Pomc *in vivo* (Takahashi et al., 1985). Thus, despite the absence of a γ-Msh domain in the *pomc* gene, the zebrafish promises to be a suitable model in further characterizing the adrenal mitogenic activity of N-Pomc that may be of particular relevance for adrenal development and growth (Yaswen et al., 1999).

The *POMC* gene is the first reported gene of adenohypophysial hormones expressed in the pituitary throughout all classes of vertebrates. In the zebrafish, *pomc* expression in pituitary corticotrophs was readily demonstrated by *in situ* hybridization as early as 24 h after fertilisation. However, using RT-PCR *pomc* expression is found even at 18 h after fertilisation. The expression of *pomc* in fertilized eggs is related to maternal expression of the gene, which disappears within hours after fertilisation. However, after 18 h a significant embryonic expression is present indicating initiation of embryonic *pomc* expression. This is supported by a study in *pomc*-GFP transgenic zebrafish embryos showing that *pomc*-GFP is expressed as early as 18-20 hpf (Liu et al., 2003a). Moreover, this study analysed the expression pattern of *pomc*-GFP in embryos also at later stages and showed that *pomc*-GFP expression is organized in distinct anterior and posterior domains of the adenohypophyse between 48 and 64 hpf (Liu et al., 2003a).

In conclusion, the availability of the structure of the *pomc* gene from zebrafish facilitates the investigation of the physiological role of the POMC-derived peptides. In particular, it is a prerequisite to clarify the role of ACTH and N-terminal POMC peptides in adrenal development.

4.2 The developmental program of zebrafish interrenal organ share some main features with that of higher vertebrates

The study on zebrafish interrenal organogenesis in this work has greatly extended that of limited previous work (Chai et al., 2003; Hsu et al., 2003). Here interrenal development was investigated by additional key steroidogenic markers (*star, mc2r*) and by further clarifying the developmental steps of the steroidogenic and also the chromaffin components.

Consistent with previous reports (Chai et al., 2003; Hsu et al., 2003) zebrafish interrenal primordia are first visualised as bilateral clusters of cells expressing *ff1b*, the teleost homologue of mammalian *SF1*. These clusters are derived from the lateral intermediate mesoderm ventral to the 3rd somite and migrate medially to form a single cell mass (Hsu et al., 2004). In wild-type zebrafish expression of steroidogenic genes like *cyp11a1* and *star* become detectable only after fusion of these two clusters. However, it has been previously shown in zebrafish mutants with midline defects that fusion of the interrenal primordia is not a prerequisite for initiation of steroidogenesis (Hsu et al., 2003). The temporal pattern of expression of steroidogenic markers in interrenal cells indicates stepwise maturation of steroidogenic cells. The sequence of gene expression resembles the findings in mammals: in mice the adrenocortical primordium is earliest visualised via expression of *Sf1* at embryonic day 9 (E9) followed by expression of steroidogenic enzymes which become detectable at E11 (Keegan and Hammer, 2002).

Only limited data have been reported on the development of the chromaffin component of the zebrafish interrenal organ (Chai et al., 2003). This work shows the expression pattern of the chromaffin marker gene dopamine β hydroxylase (*dβh)* in relation to that of steroidogenic marker throughout development. Although being detected in the embryo already at 22 hpf, *dβh* expression is initially visualised in the interrenal primordium at 2dpf. Intriguingly *dβh* expressing chromaffin cells initially overlap with the steroidogenic primordium on one side only. Chromaffin cells then converge to the midline and fuse at 3 dpf. From this stage onwards steroidogenic and chromaffin cells remain in close contact and accordingly expand together bilateral to the notochord. At 5-7 dpf chromaffin cells

appear to form a central compartment of the interrenal organ. Again, the co-development of steroidogenic and chromaffin cells resembles the organogenesis of the adrenal gland in mammals. In mice neural crest-derived chromaffin cells migrate into the adrenocortical primordium only at E12-E14 to form cell clusters that later coalesce to a distinct layer in the center of the organ (Keegan and Hammer, 2002). Thus zebrafish interrenal organogenesis consists of conserved features in the developmental program with respect to the sequential expression of specific homologous genes as well as cell migration.

4.3 Functional interaction of steroidogenic and chromaffin cells of the interrenal organ

In mammals, adrenal cortex and medulla have been shown to have bidirectional functional interaction, as hormone products of the cortex can affect medullary catecholamine secretion and vice versa (Ehrhart-Bornstein et al., 1998; Unsicker et al., 2005). However, the role of glucocorticoids in determination and development of chromaffin medullary cells remains to be further elucidated (Unsicker et al., 2005). This work gives indirect but convincing evidence that glucocorticoids are essential for chromaffin cell differentiation.

As only limited interrenal chromaffin markers in zebrafish are available, interrenal chromaffin development and differentiation was assayed here by expression analysis of the chromaffin marker gene *dβh*. The results show that the expression pattern of *dβh* is unchanged in the *aal/eya1* and *lia/fgf3* mutant embryos lacking pituitary/Pomc pituitary at 2 dpf when expression of the steroidogenic marker genes is also unaffected. Later, at 5 dpf, when the expression of steroidogenic markers is significantly decreased, a remarkable decrease in chromaffin *dβh* gene expression concerning both area and density is also observed in these mutants. The expression of *dβh*, however, is unchanged in the pituitary mutant *pit1* having corticotrophs and consequently normal expression of steroidogenic markers at 5 dpf. Moreover, this expression pattern of chromaffin *dβh* gene is also observed in *mc2r* knockdown embryos at 2 and 5 dpf in the same manner as in *aal/eya1* and *lia/fgf3* mutants. These results provide an indirect evidence that lack of pituitary corticotrophs/Pomc or lack of Mc2r-signalling leads to a decrease in the expression of steroidogenice genes and consequently to glucocorticoid deficiency (see also below) that

reduces the chromaffin marker gene *dβh* transcripts. The reduced area of chromaffin cells provides also indirect evidence that glucocorticoids may play a role in the early stage of chromaffin cell development. This is further supported by data derived from DEX treated embryos at 5dpf. These embryos have no anterior pitutary *pomc* expression due to DEX suppression but exhibit quite normal *dβh* expression. The data here are in accordance with *in vivo* data gained in hypophysectomized rats (Wurtman and Axelrod, 1966), in CRH-R1 -/- mice (Yoshida-Hiroi et al., 2002) and in 21-hydroxylase-deficiency mice (Bornstein et al., 1999). In these animal models, expression of chromaffin marker *Dβh* was not assayed, but a decrease in activity of phenylethanolamine N- methyltransferase, another marker of chromaffin enzyme activity catalyzing the N-methylation of noradrenaline to adrenaline was observed. This enzyme activity was restored by ACTH or dexamethasone injection in hypophysectomized rats. Moreover, reduced medullary catecolamine secretion as well as altered chromaffin cell migration, development and structure were also reported in the 21-hydroxylase-deficient mice (Bornstein et al., 1999).

However, from our investigations it remains inconclusive whether glucocorticoids only or also steroidogenic cells per se can affect determination and development of chromaffin cells. Finotto et al. showed that lack of glucocorticoid receptors in *Gr* -/- mice does not affect medullary development except expression of PNMT while in *Sf1*-/- mice lacking the entire adrenal cortex only half of the chromaffin cells were found, with normal characteristics except the lack of PNMT expression (Finotto et al., 1999) indicating that beside glucocorticoids, cortical cues may be required for proper migration of chromaffin cells to the adrenal anlage. The avaibility of the zebrafish model should facilitate experiments to settle this issue.

4.4 Pituitary-interrenal interactions in early stages of zebrafish development

4.4.1 Role of pituitary/Pomc derivates in interrenal development

In this study the role the pituitary in the development of the zebrafish interrenal organ was analysed. It was demonstrated here that after an early phase of pituitary-independent

development and steroidogenic enzyme activity pituitary corticotrophs are required for full functional differentiation of the interrenal organ acting via interrenal mc2 receptors.

Our results document that early interrenal development is fully independent of any pituitary influence. In fact, steroidogenic enzyme expression as assessed by *3β-Hsd* enzyme activity precedes expression of the *mc2r* giving indirect evidence of early autonomous pituitary-independent steroidogenesis. At 2dpf, expression of interrenal markers is not affected in pituitary mutants lacking *pomc* expressing cells, nor in *mc2r* knockdown embryos, or embryos treated with exogenous dexamethasone. Thus, although *pomc* expression in wild-type zebrafish is earliest detectable at 18 hpf (Hansen et al., 2003; Liu et al., 2003a) and, therefore, precedes interrenal *ff1b* expression, its effects on the interrenal steroidogenic component are delayed and control of steroid hormone production by pituitary *Pomc* requires further maturation of the interrenal tissue. Autonomous interrenal steroidogenesis was also described in common carp and rainbow trout during developmental stages from the time when endogenous cortisol secretion begins to the time a cortisol response to exogenous stressors is observed (36-50 hpf in common carp and week 1^{st}-2^{nd} after hatching in rainbow trout (Stouthard,1998, Barry, 1995)). From these data, however, it was concluded that functional intergration of the HPI axis depends on maturation of the brain, hypothalamic, or sensory components of the organism (Barry, 1995), whereas our results suggest that maturation of interrenal tissue itself is of major important.

Our findings are in agreement with adrenal development in *Pomc*-null mice, as these mice are born with adrenal glands that are morphologically indistinguishable from those of their wild-type littermates (Karpac et al., 2005). Only postnatally in *Pomc*-null mice adrenal cells fail to proliferate and gradually develop atrophy (Karpac et al., 2005). Moreover, the results of pituitary-independent early interrenal development are resembling findings in human anencephalic fetuses that do not have a pituitary: in early gestation (before weeks 10-15), adrenal development of anencephalic fetuses is normal. Only thereafter the fetal zone fails to develop and does not exhibit its characteristic growth and steroidogenic activity (Mesiano and Jaffe, 1997). However, different from our mutants, *Pomc*-null mice lack all *POMC* transcripts and anencephalic fetuses also lack the hypothalamus. Thus, with regard to adrenal development, mice lacking the transcription factor TPIT essential for

development of pituitary corticotrophs, or hypophysectomized animals seem to be more comparable to zebrafish *aal/eya1* and *lia/fgf3* mutants. In *Tpit* -/- mice, adrenals are detectable but hypoplastic, with significant loss in the glucocorticoid-producing zona fasciculata. Similar to *Pomc*-null mice corticosterone is undetectable suggesting dependence on pituitary POMC of both adrenal growth and corticosterone secretion (Pulichino et al., 2004; Pulichino et al., 2003). However, the age of the mice in these studies was not given and it is likely that these data were gained not in fetal embryos or newborn mice but in adult *Tpit* -/- animals. Thus, data on fetal and neonatal *Tpit* -/- mice are needed to assess the specific influence of pituitary corticotrophs on prenatal development of the adrenal gland. Results obtained in hypophysectomized fetal sheep and pigs have demonstrated that hypophysectomy inhibits the intrauterine growth of the adrenal cortex, particularly of the zona fasciculata (Coulter et al., 1989; Nicolle and Bosc, 1989; Nicolle and Bosc, 1990). However, in these models analysis is restricted to later stages of gestation, thereby precluding analysis of early loss of corticotroph function.

The understanding of pituitary-independent early steroidogenesis is incomplete but may be of clinical relevance, as it is the hallmark of adrenal Cushing's syndrome. Thus, autonomous POMC-independent cortisol production in adrenal tumors may be the result of adrenal reprogramming towards an early developmental phenotype. In zebrafish Ff1b is clearly required for early interrenal steroidogenesis, as *ff1b* knockdown not only leads to downregulation of *cyp11a1* and *3β-hsd* but eventually also to loss of steroidogenic tissue (Chai et al., 2003; Hsu et al., 2003). More recently, an important role for the interaction of the transcription factor Prox1 with Ff1b has been reported, as *prox1* morphants display loss of *ff1b* expression and 3β-Hsd activity (Liu et al., 2003b). In addition, the transcription factor Wt1 has been shown to be involved in early zebrafish interrenal development, as reduced *wt1* levels in knockdown experiments led to smaller interrenal primordia and decreased *ff1b* expression (Hsu et al., 2003). The pivotal role of Ff1b for early pituitary independent steroidogenesis is also evident from experiments demonstrating direct activation of *cyp11a1* transcription similar to its mammalian counterpart *SF1* (Hsu et al., 2003). On the other hand, most recently expression of *cyp11a1* has been described in the extra-embryonic yolk syncytial layer of zebrafish embryos, converting cholesterol to pregnelonone and playing a major role in embryonic cell movement and stabilization of

microtubules (Hsu et al., 2006b). Intriguingly this expression is seemingly independent of Ff1b suggesting that Ff1b is not an invariable prerequisite for steroidogenic activity of Cyp11a1 in zebrafish.

The investigations in zebrafish at 5 dpf clearly indicate that at this stage interrenal development and function has become dependent on pituitary signals. In both *aal/eya1* and *lia/fgf3* mutants not only the expression of steroidogenic markers was decreased indicating lower functional activity but also the area of expression was reduced suggesting of interrenal hypoplasia. Hammerschmidt et al. (Nica et al., 2004) have suggested that at 5dpf zebrafish development largely resembles the developmental stage at birth in mammals. In human anencephalic fetuses at late gestation, adrenal hypoplasia with a strongly reduced fetal zone has been described (Bocian-Sobkowska et al., 1997a; Bocian-Sobkowska et al., 1997b) suggesting that in humans regulation of the adrenal by the pituitary gland is, at least in part, established prior to birth.

As *aal/eya1* and *lia/fgf3* mutants lack multiple pituitary cell types, the interrenal phenotype in zebrafish could be the result of multiple hormonal deficiencies. However, the findings in *pit1* mutants clearly suggest that the presence of corticotrophs is sufficient for normal interrenal development at 5 dpf indicating that pituitary Pomc secretion by corticotrophs is the essential signal. Furthermore, *mc2r* morphants exhibit a similar phenotype as mutants lacking pituitary corticotrophs suggesting that Acth signalling is crucial for the action of pituitary corticotrophs on interrenal development at 5 dpf. This is in keeping with the observation that the adrenal phenotype in patients with ACTH resistance due to inactivating *MC2R* mutations, familial glucocorticoid deficiency type 1, resembles the findings in anencephalic fetuses (Berberoglu et al., 2001). As *Mc2r*-null mouse mutants have not yet been generated, no direct comparison with the phenotype in zebrafish is possible. The role of other POMC derived peptides for the development of steroidogenic cells remains a matter of debate. There is substantial evidence that N-terminal POMC derived peptides possess mitogenic activity in the adrenal cortex and may be involved in adrenal proliferation (Estivariz et al., 1988a; Estivariz et al., 1982; Estivariz et al., 1992; Estivariz et al., 1988b; Fassnacht et al., 2003). However, while it was demonstrated that 1-28 N-POMC induces cell proliferation in adrenal cells *in vitro*, administration of 1-28 N-

POMC in *Pomc*-null mice and *Tpit* -/- mice so far failed to affect adrenal growth (Coll AP, 2006). Even so, it is possible that different dosing or other N-POMC derived peptides including glycosylated forms may result in more biological activity of the exogenously administered N-POMC. Also zebrafish Pomc contains a highly conserved homologue of the N-terminus of N-Pomc. In addition, it has been reported that N-Pomc derived peptides can slightly enhance Acth-induced cortisol release in teleosts (Takahashi et al., 1985). Thus, a role of N-Pomc derived peptides for growth of interrenal cells in zebrafish cannot be fully excluded. Nevertheless, our findings clearly indicate that intact Mc2r signalling is a prerequisite for such a role.

The suppression of endogenous *pomc* secretion by exogenous dexamethasone in wild-type zebrafish indicates that a pituitary influence is initiated as early as at 3 dpf, as at this stage a reduction in steroidogenic markers commences and becomes progressively more pronounced until 5 dpf.

Mc2r morphants facilitated analysis of feedback control of reduced steroidogenesis at the pituitary level. As anticipated no effect of *mc2r* knockdown on pituitary *pomc* expression is found at 2 dpf, as steroidogenesis is not yet affected. However, in response to decreased Mc2r-dependent steroidogenesis, pituitary *pomc* expression was strongly upregulated at 5 dpf. This increase in *pomc* expression is restricted to the anterior domain of the pituitary gland indicating that only this compartment of pituitary *pomc* expressing cells is involved in the control of interrenal steroidogenesis. Accordingly, exogenous dexamethasone only reduces *pomc* expression in this domain, whereas otherwise *pomc* expression remains unchanged. These findings are in agreement with the report by Liu et al. using transgenic zebrafish expressing green fluorescent protein (GFP) driven by the *pomc* promoter (Liu et al., 2003a). In these zebrafish GFP was mainly targeted to anterior and posterior corticotrophs, whereas MSH-positive cells located slightly anterior of the posterior corticotrophs were GFP-negative (Liu et al., 2003a). Consistent with our data, glucocorticoids only suppressed *pomc*-GFP transgene expression in anterior corticotrophs, but not in posterior corticotrophs, indicating that these cells are regulated differently.

4.4.2 Functional ontogeny of the pituitary-interrenal axis and glucocorticoid feedback regulation in zebrafish

The suppression of endogenous *pomc* expression by exogenous dexamethasone in wild-type zebrafish indicates that a pituitary influence is initiated as early as at 3 dpf, as at this stage a reduction in steroidogenic markers commences and becomes progressively more pronounced until 5 dpf. This is also an indirect evidence that the initial functional integration of the HPI axis in zebrafish occurs at 3 dpf, at hatching time, as reported in common carp, the closest teleost to zebrafish, although in some other teleosts, normal HPI function was reported only 1-2 weeks after hatching (Stouthart et al., 1998). Moreover, the data gained in zebrafish resemble those described in mammals, as the mammalian HPA axis starts to function in late fetal life (Kapoor et al., 2006).

The studies using exogenous DEX clearly demonstrate glucocorticoid feedback at 2 dpf and, therefore, indicate that negative feedback at the pituitary level precedes initiation of the control of interrenal steroidogenesis by the anterior domain of pituitary corticotrophs. Negative feed back regulation of glucocorticoid at different levels of HPA axis has also been described in fetal life in sheep and guinea pigs and mice (Matthews and Challis, 1995; McCabe et al., 2001; Reichardt and Schutz, 1996; Unno et al., 1998)**.** However, whether a similar sequential pattern of the development of the feedback loop of the pituitary adrenal axis is also operating in mammals is not yet known.

Taken together, our findings demonstrate that interrenal development in the zebrafish shares many conserved molecular and developmental mechanisms with higher vertebrates. Thus zebrafish is a highly suitable model organism to further investigate the roles of transcription factors involved in human and mouse adrenal development. Moreover, with the availability of robust and specific markers of steroidogenic cells like *star* and *cyp11a1* zebrafish mutagenesis screens can be now used to detect mutations associated with early interrenal hypoplasia or even agenesis. Such mutants may eventually allow identification of new genes involved in early adrenal development in higher vertebrates and humans.

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6 Appendix

6.1 Primers used to clone zebrafish *pomc* gene

6.1.1 *pomc* gene specific primers *:*

GSP1 (forward):

5'-CAG AGA TGG TGA GGG GAG TGA GGA TGT TGT GT-3'

GSP2 (reverse):

5`- CTT AAA GCC ACT CAC TCA TCC TTC CTC GGT TG-3'

GSP3 *(reverse,* specific primer for 5'-RACE-PCR):

5'-TCA CTC ATC CTT CCT CGG TTG GTC TTT ATG CAT TAC GTT-3'

GSP4 *(forward,* specific primer for 3'-RACE-PCR):

5'-ATG GCT TTG TCC TTG CCT CCT CGT CCT GCC CT-3'

6.1.2 The primers provided with SMART ^{TM} RACE cDNA Amplification Kit

SMART II A Oligonucleotide (10µM) :

5'- AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'

3'-CDS (3' - RACE CDS Primer A, 10µM) :

5'-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}N_{-1}$N-3'

(N= A,C,G, or T; N_{-1} = A, G, or C)

5'-CDS (5'- RACE CDS Primer A,10µM):

5' – $(T)_{25}N_{-1}$N -3'

(N= A,C,G, or T; N_{-1} = A, G, or C)

UPM (10x Universal Primermix A)

Long (0.4µM) :

5'- CTA ATA CGA CTC ACT ATA AGG GCA AGC AGT GGT ATC AAC GCA GAG T-3'

short (2µM) :

5'- AAGCAGTGGTATCAACGCAGAGT-3'

6.2 primers used for RT-PCR to analyse the expression of *pomc*-gene during zebrafish embryonic development:

GSP5 (*Danio rerio pomc* forward) :

5'-ATG GTG AGG GGA GTG AGG ATG TTG TGT-3'

GSP6 (*Danio rerio pomc* reverse):

5'-CCA CTC ACT CAT CCT TCC TCG GTT G - 3'

GSP7 (*Danio rerio* β-actin forward):

5'-TTC AAC AGC CCT GCC ATG TA-3'

GSP8 (*Danio rerio* ß-actin reverse):

5'-GCA GCT CAT AGC TCT TCT CCA GGG AG-3'

All the gene specific primers were supplied by thermo Hybaid, Ulm, Germany and used with the concentration of 10pM/µl

6.3 Table

Tab. 3: proteinase K dilution from the stock solution of 2mg/ml for proteinase K (pnK) treatment in ISH

Stages of embryos (hpf)	Dilution of pnK from 2mg/ml stock solution	Time of digestion (min)
24	1:1000	6
26	1:1000	8
28	1:1000	10
30	1:1000	30
32/33	1:500	25
35	1:250	10
40	1:250	15
45	1:250	30
48	1:250	35
55	1:250	45
60	1:100	30
72-75	1:100	60
80-100	1: 50	60
120	1:35	60
168	1:25	60

Publications

Hansen, I. A., To, T. T., Wortmann, S., Burmester, T., Winkler, C., Meyer, S. R., Neuner, C., Fassnacht, M. and Allolio, B. (2003), *The pro-opiomelanocortin gene of the zebrafish (Danio rerio,* Biochem Biophys Res Commun 303, 1121-8.

Thuy Thanh To, Stefanie Hahner, Gabriela Nica, Klaus Rohr, Matthias Hammerschmidt, Christoph Winkler, and Bruno Allolio, *Pituitary-interrenal interaction in zebrafish interrenal organ development,* submitted to Molecular Endocrinology (accepted for publication pending minor revisions).

Scientific communications

To Thanh Thuy, Immo Alex Hansen, Sebastian Wortmann, Susanne R. Meyer, Stefanie Hahner, Christoph Winkler, Martin Fassnacht, Bruno Allolio, C*harakterisierung des POMC-Gens des Zebrafisches (Danio rerio*), 3rd scientific symposium of the Department of Medicine, University of Wuerzburg , Bad Brückenau, Germany, March 2003 (poster presented by TTT).

M. Fassnacht, I.A. Hansen, T.T.To, C. Winkler, T.Burmester, C.Neuner, S.Mayer, B. Allolio, *Proopiomelanocortin (POMC) in the Zebrafish (Danio rerio)-Complete sequence, phylogenetic evolution and expression* Exp Clin Endocrinol Diabetes 2003 111(suppl 1):V68 (oral presentation presented by MF).

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Lebenslauf

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Fremdsprachen: Englisch, Deutsch

Eklärung

Die vorliegende Arbeit wurde von mir selbständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe früher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, August 2006

Thuy Thanh To