TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 852

MEDICA - ODONTOLOGICA

THE EFFECTS OF ENDOCRINE DISRUPTERS ON FETAL MALE RAT TESTICULAR AND ADRENAL DEVELOPMENT

by

Annika Adamsson

TURUN YLIOPISTO
Turku 2009

From the Departments of Physiology and Paediatrics
and the Laboratory of Electron Microscopy
University of Turku
Turku, Finland

Supervised by Professor Jorma Toppari, MD, PhD
Departments of Physiology and Paediatrics
University of Turku
Turku, Finland

and

Docent Jorma Paranko, PhD
Department of Cell Biology and Anatomy
University of Turku
Turku, Finland

Reviewed by Professor Raimo Pohjanvirta, DVM, PhD
Department of Food and Environmental Hygiene
University of Helsinki
Helsinki, Finland

and

Docent Krista Erkkilä, MD, PhD
Children's Hospital
University of Helsinki
Helsinki, Finland

Dissertation opponent Professor Ulf Magnusson, DVM, PhD
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences
Uppsala, Sweden

Cover Erika Adamsson,"Suburban landscape", part of the series 2009
Oil on plexiglass

ISBN 978-951-29-3933-6 (PRINT)
ISBN 978-951-29-3934-3 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

ABSTRACT

Annika Adamsson

The effects of endocrine disrupters on fetal male rat testicular and adrenal development

Departments of Physiology and Paediatrics and the Laboratory of Electron Microscopy, University of Turku, Finland
Annales Universitatis Turkuensis
Painosalama Oy, Turku, Finland 2009

Many of the reproductive disorders that emerge in adulthood have their origin during fetal development. Numerous studies have demonstrated that exposure to endocrine disrupting chemicals can permanently affect the reproductive health of experimental animals. In mammals, male sexual differentiation and development are androgen-dependent processes. In rat, the critical programming window for masculinization occurs between embryonic days (EDs) 15.5 and 19.5. Disorders in sex steroid balance during fetal life can disturb the development of the male reproductive tract. In addition to the fetal testis, the adrenal cortex starts to produce steroid hormones before birth. Glucocorticoids produced by the adrenal cortex are essential for preparing the fetus for birth. In the present study, the effects of exposure to endocrine disrupters on fetal male rat testicular and adrenal development were investigated. To differentiate the systemic and direct testicular effects of endocrine disrupters, both *in vivo* and *in vitro* experiments were performed. The present study also clarified the role of desert hedgehog signalling (Dhh) in the development of the testis.

The results indicate that endocrine disrupters, diethylstilbestrol (DES) and flutamide, are able to induce rapid steroidogenic changes in fetal rat testis under *in vitro* conditions. Although *in utero* exposure to these chemicals did not show overt effects in fetal testis, they can induce permanent changes in the developing testis and accessory sex organs later in life. We also reported that exposure to antiandrogens can interfere with testicular Dhh signalling and result in impaired differentiation of the fetal Leydig cells and subsequently lead to abnormal testicular development and sexual differentiation. *In utero* exposure to tetrachlorodibenzo-*p*-dioxin (TCDD) caused direct testicular and pituitary effects on the fetal male rat but with different dose responses. In a study in which the effects of developmental exposure to environmental antiandrogens, di-isononylphthalate and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), on fetal male rat steroidogenesis were investigated, chemicals did not down-regulate testicular or adrenal steroid hormone synthesis or production in 19.5-day-old fetal rats. However, *p,p'*-DDE-treatment caused clear histological and ultrastructural changes in the prenatal testis and adrenal gland. These structural alterations can disturb the development and function of fetal testis and adrenal gland that may become evident later in life.

Exposure to endocrine disrupters during fetal life can cause morphological abnormalities and alter steroid hormone production by fetal rat Leydig cells and adrenocortical cells. These changes may contribute to the maldevelopment of the testis and the adrenal gland. The present study highlights the importance of the fetal period as a sensitive window for endocrine disruption.

Key words: rat, fetus, testis, adrenal, endocrine disrupter, steroidogenesis, development

TIIVISTELMÄ

Annika Adamsson

Hormonaalisten haitta-aineiden vaikutukset rottasikiön kiveksen ja lisämunuaisen kehitykseen

Fysiologia, Lastentaudit ja Elektronimikroskopian laboratorio,
Turun yliopisto
Annales Universitatis Turkuensis
Painosalama Oy, Turku, 2009

Monet lisääntymishäiriöt saavat alkunsa sikiökautisen kehityksen aikana. Lukuisat tutkimukset ovat osoittaneet, että hormonaaliset haitta-aineet voivat pysyvästi vahingoittaa koe-eläinten lisääntymisterveyttä. Nisäkkäillä koiraiden sukupuolinen erilaistuminen ja sukupuolen mukainen kehitys ovat androgeeneista riippuvaisia tapahtumia. Sukupuolielimistön maskulinisaatio tapahtuu rotalla 15,5-19,5 raskauspäivien välisenä aikana. Häiriöt sikiökautisissa sukupuolihormonitasoissa voivat häiritä koiraan normaalia sukupuolielimistön kehitystä. Kiveksen lisäksi myös lisämunuaiskuori tuottaa steroidihormoneja ennen syntymää. Lisämunuaiskuoren glukokortikoidi-tuotanto on välttämätöntä sikiön valmistautuessa syntymään. Tässä väitöskirjatutkimuksessa olen tutkinut hormonaalisten haitta-aineiden vaikutuksia rottasikiön kiveksen ja lisämunuaisen kehitykseen. Selvittääkseni hormonaalisten haitta-aineiden systeemisiä ja suoria kivesvaikutuksia, tein sekä *in vivo* että *in vitro* -kokeita. Tutkimuksessa selvitin myös Desert hedgehog (Dhh) -signaloinnin merkitystä kiveksen kehitykseen.

Tulokset osoittavat, että hormonaaliset haitta-aineet, dietyylistilbestroli (DES) ja flutamidi, saavat aikaan nopeita steroidogeneesin muutoksia rottasikiön kiveksessä *in vitro* -olosuhteissa. Raskauden aikainen *in utero* -altistus DES:lle ja flutamidille voi myös aiheuttaa pysyviä muutoksia kehittyvään kivekseen ja lisäsukupuolirauhasiin. Emme kuitenkaan havainneet hormonituotannon muutoksia vielä sikiöaikana näillä kemikaaleilla. Sen sijaan havaitsimme, että antiandrogeenialtistus voi häiritä kiveksen Dhh-signalointia ja heikentää sikiön Leydigin solujen erilaistumista, mikä johtaa kiveksen epänormaaliin kehitykseen ja heikentyneeseen sukupuoliseen erilaistumiseen. Raskauden aikainen altistus 2,3,7,8-tetrakloori-dibentso-*p*-dioksiinille (TCDD) aiheutti suoria kives- ja aivolisäkevaikutuksia rottasikiökoirailla, mutta erilaisella annosvasteella. Tutkimus, jossa selvitimme antiandrogeenisten ympäristömyrkkyjen, di-isononyyliftalaatin ja 1,1-dikloori-2,2-bis(*p*-kloorifenyyli)-etyleenin (*p,p'*-DDE:n), raskauden aikaisen altistuksen vaikutuksia 19,5 päivän ikäisen rottasikiön steroidogeneesiin osoitti, että näillä kemikaaleilla ei ollut vaikutusta sikiön kiveksen tai lisämunuaisen steroidihormonien synteesiin tai tuotantoon. Altistus *p,p'*-DDE:lle aiheutti kuitenkin selkeitä muutoksia sekä sikiön kiveksen että lisämunuaisen hienorakenteeseen. Nämä rakenteelliset muutokset saattavat häiritä sikiön kiveksen ja lisämunuaisen myöhempää kehitystä ja toimintaa.

Sikiökautinen altistuminen hormonaalisille haitta-aineille voi aiheuttaa rakenteellisia muutoksia ja muuttaa steroidihormonien tuotantoa rottasikiön Leydigin soluissa ja lisämunuaiskuoren soluissa. Nämä muutokset saattavat johtaa kiveksen ja lisämunuaisen häiriintyneeseen kehitykseen. Tämä tutkimus tuo esiin sikiökauden merkityksen herkkänä ajanjaksona hormonaaliselle häirinnälle.

Avainsanat: rotta, sikiö, kives, lisämunuainen, hormonaalinen haitta-aine, steroidogeneesi, kehitys

TABLE OF CONTENTS

ABSTRACT **3**

TIIVISTELMÄ **4**

TABLE OF CONTENTS **5**

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **10**

1 INTRODUCTION **11**

2 REVIEW OF THE LITERATURE **12**

2.1 Testis determination in mammals 12

2.2 Testis development 13

2.2.1 Desert hedgehog signalling in the fetal Leydig cell 15

2.3 Development of mammalian adrenal glands 16

2.4 Maturation of hypothalamus-pituitary-adrenal/gonadal (HPA/HPG) function 19

2.5 Steroidogenesis 20

2.6 Testicular dysgenesis syndrome (TDS) 22

2.7 Endocrine disrupters 23

2.7.1 Diethylstilbestrol 24

2.7.2 Flutamide 25

2.7.3 Di-isononylphthalate 26

2.7.4 DDT and *p,p'*-DDE 27

2.7.5 Dioxins and TCDD 28

2.7.5.1 AhR and the mechanism of action 30

3 AIMS OF THE PRESENT STUDY **32**

4 MATERIALS AND METHODS **33**

4.1 Animals 33

4.2 *In utero* exposure and the study design for developmental analysis 33

4.3 *In vitro* organ cultures 34

4.4 Sample collection 34

4.5 Real-time quantitative RT-PCR 35

4.6 Hormone measurements 35
4.6.1 LH 35
4.6.2 Corticosterone 35
4.6.3 Progesterone and testosterone 36
4.7 Western blot analysis 36
4.8 Immunohistochemistry 37
4.9 Light and electron microscopy 37
4.10 Statistical analysis 38
5 RESULTS 39
5.1 Developmental studies 39
5.1.1 The ontogeny of fetal testicular steroidogenesis and plasma LH (II) 39
5.2 *In utero* exposure to endocrine disrupters 39
5.2.1 Toxicity of chemicals (I-IV) 39
5.2.2 Effects on plasma hormone levels (II-III) 39
5.2.3 mRNA and protein expression levels of steroidogenic enzymes and related factors in fetal testis (I, III, IV) 39
5.2.3.1 mRNA expression of genes involved in desert hedgehog signalling (II) 40
5.2.4 Effects on intratesticular testosterone and progesterone levels (I, III, IV) 40
5.2.5 mRNA and protein expression levels of steroidogenic enzymes and related factors in fetal adrenals (III-IV) 41
5.2.6 Effects on testicular and adrenal histology and ultrastructure (IV) 42
5.3 Testicular organ cultures and exposure to endocrine disrupters 42
5.3.1 The effects of diethylstilbestrol on testicular steroidogenesis (I) 42
5.3.2 The effects of flutamide on testicular steroidogenesis (I) 43
5.3.3 The direct effects of Dhh signalling on testicular development 43
6 DISCUSSION 45
6.1 Testicular steroidogenesis during prenatal life 45
6.2 Critical developmental window for exposure to endocrine disrupters 45
6.3 Comparison of *in vivo* and *in vitro* exposure 48
6.4 Desert hedgehog signalling in developing rat testis 49
7 CONCLUSIONS 51
8 ACKNOWLEDGEMENTS 52

REFERENCES 54

ORIGINAL PUBLICATIONS I – IV 65

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AFP	alpha fetoprotein
AGD	anogenital distance
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor suppressor
AMH	anti-Müllerian hormone
AR	androgen receptor
ARKO	androgen receptor knock-out
ARNT	aryl hydrocarbon receptor nuclear translocator
BAX	Bcl-2 associated X gene
BBP	benzyl butyl phthalate
BrdU	5-bromo-2-deoxyuridine
CAH	congenital adrenal hyperplasia
cDNA	complementary DNA
CRH	corticotropin releasing hormone
CV	coefficient variation
CYP1	cytochrome P450, family 1
CYP1A1	cytochrome P450 1A1
DAX1	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBP	dibutyl phthalate
DDT	1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane
DEHP	diethylhexyl phthalate
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHH	desert hedgehog
DHT	dihydrotestosterone
DINP	di-isononylphthalate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ED	embryonic day
EMX2	empty spiracles homeobox 2
ERα	estrogen receptor alpha
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GLI	glioma-associated oncogene
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
Hh	hedgehog signalling
HOX genes	homeotic genes
HPA axis	hypothalamus-pituitary-adrenal axis

HPG axis	hypothalamus-pituitary-gonadal axis
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
HSP	heat shock protein
INSL3	insulin-like factor 3
LD_{50}	lethal dose 50 %
LH	luteinizing hormone
LIM1	LIM homeobox gene1
LIM9	LIM homeobox gene 9
mRNA	messenger ribonucleic acid
o,p'-DDD	1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane; mitotane
P4	progesterone
P450aldo	aldosterone synthase cytochrome P450
P450arom	cytochrome P450 aromatase
P450c11	cytochrome P450 11β-hydroxylase
P450c17	cytochrome P450 17α-hydroxylase/17-20 lyase
P459c21	cytochrome P450 21-hydroxylase
P450scc	cytochrome P450 cholesterol side chain cleavage
PBS	phosphate-buffered saline
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PE	phthalate ester
PMSF	phenylmethylsulphonyl fluoride
PND	postnatal day
p,p'-DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
PTCH1	patched 1
PTCH2	patched 2
PVC	polyvinyl chloride
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of mean
SF1	steroidogenic factor 1
SHBG	sex hormone binding globulin
SMO	smoothened
SOX9	SRY-related high-mobility group (HMG) box 9
StAR	steroidogenic acute regulatory protein
SRY	sex-determining region of the Y chromosome
SUFU	suppressor of fused
T	testosterone
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDS	testicular dysgenesis syndrome
WNT4	wingless-type MMTV integration site family, member 4
WT1	Wilms' tumor suppressor gene 1
XRE	xenobiotic response element

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by the Roman numerals I-IV.

I. Adamsson Annika*, Brokken Leon*, Paranko Jorma, Toppari Jorma (2008). *In vivo* and *in vitro* effects of flutamide and diethylstilbestrol on fetal testicular steroidogenesis in the rat. *Reproductive Toxicology* 25: 76–83. ** Shared first author*.

II. Brokken Leon*, Adamsson Annika*, Paranko Jorma, Toppari Jorma (2009). Anti-androgen exposure *in utero* disrupts expression of desert hedgehog and insulin-like factor 3 in the developing fetal rat testis. *Endocrinology* 150: 445-451. ** Shared first author.*

III. Adamsson Annika, Simanainen Ulla, Viluksela Matti, Paranko Jorma, Toppari Jorma (2008). The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on foetal male rat steroidogenesis. *International Journal of Andrology.* E-pub ahead of print.

IV. Adamsson Annika, Salonen Virpi, Paranko Jorma, Toppari Jorma (2009). Effects of maternal exposure to di-isononylphthalate (DINP) 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) on steroidogenesis in the fetal rat testis and adrenal gland. *Reproductive Toxicology.* Accepted for publication.

1 INTRODUCTION

Many xenobiotics and naturally occurring compounds have been found to disrupt the endocrine system of experimental animals and wildlife. The term endocrine disrupter is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (International Programme on Chemical Safety; http://whqlibdoc.who.int/hq/2002/a76784_ch1.pdf). In mammals, the development of the male phenotype is critically dependent on androgens produced by fetal Leydig cells. In rat, the critical programming window for masculinization occurs between embryonic days (EDs) 15.5 and 19.5 (Welsh *et al*. 2008). Disorders in sex steroid balance during fetal development may interfere with male reproductive success and health later in life (Sharpe 2001). Concern about the impact of endocrine disrupters on human health has arisen from studies which have shown adverse trends in male reproductive health; decreased semen quality (Carlsen *et al*. 1992) and increased incidence of hypospadias and cryptorchidism (Matlai & Beral 1985) have been reported in humans.

Chemicals with estrogenic or antiandrogenic properties can affect the development of androgen-sensitive tissues in males. Exposure to endocrine disrupters during fetal development typically suppresses male sexual development and is associated with reproductive tract malformations, cryptorchidism and hypospadias (Sharpe 2001). A variety of testicular disorders observed may result from impaired development of fetal Leydig and Sertoli cells. However, to date the molecular mechanisms underlying these observations have not been well characterized.

In addition to the testis, the adrenal cortex becomes steroidogenically active before birth. Adrenocorticotrophic hormone (ACTH) that stimulates the secretion of glucocorticoids is also capable of stimulating fetal Leydig cell function in a similar way as luteinizing hormone (LH) (O'Shaughnessy *et al*. 2003). In the rat, corticosterone production peaks on ED 19 (Boudouresque *et al*. 1988). The prenatal peak in glucocorticoid production is essential for preparing the fetus for birth (Liggins 1994).

In the present study, we investigated the effects of exposure to endocrine disrupters on fetal male rat testicular and adrenal development. To differentiate the systemic and direct testicular effects of endocrine disrupters, *in vitro* cultures were also performed. The present study also clarifies the role of desert hedgehog signalling (Dhh) in the development of the testis.

2 REVIEW OF THE LITERATURE

2.1 TESTIS DETERMINATION IN MAMMALS

Genetic sex is established at fertilization with the inheritance of paternal and maternal haploid gametes. After the determination of genetic sex, indifferent gonads, located adjacent to the mesonephroi, will develop into either testes or ovaries. Indifferent gonads are paired structures that arise from intermediate mesoderm forming the urogenital ridge that is further divided into the adrenogonadal primordium and the attached mesonephros. Thus the urogenital ridge contributes lineages to the adrenal cortex, gonads and kidney (Keegan & Hammer 2002, Parker & Schimmer 2002). The indifferent gonads (or genital ridge) contain both somatic cells that are of mesonephric origin and primordial germ cells that have migrated from the yolk sac endoderm (Magre & Jost 1991).

Testis determination is a highly regulated process in which several regulatory genes play a role. *The sex-determining region of the Y chromosome* (*SRY)* acts as the main determinant for testicular differentiation. In the mouse, *Sry* is expressed in the supporting cell lineage (pre-Sertoli cells) of the genital ridge in a narrow temporal window, between embryonic days (EDs) 10.5 and 12.5 (Koopman *et al*. 1990, Hacker *et al*. 1995), just prior to the appearance of the differentiated testis (Figure 1). *Sry* induces the differentiation of Sertoli cells (Jamieson *et al*. 1998, Wilhelm *et al*. 2005). It has also been shown that migration of mesonephric cells into the differentiating gonad is associated with *Sry's* presence (Capel *et al*. 1999) as well as the proliferation of the cells within the genital ridge (Schmahl *et al*. 2000). *SOX9 [SRY-related high-mobility group (HMG) box 9]*, is known to be important in the regulation of sex determination. Mutations and translocations of the human *SOX9* gene are associated with campomelic dysplasia, a disorder with severe congenital skeletal abnormalities (Foster *et al*. 1994). Many of these patients with XY karyotype also have genital defects; approximately 75 % of them suffer from sex reversal and other genital disorders (Hovmoller *et al*. 1977, Foster *et al*. 1994, Schafer *et al*. 1996). In mice, *Sox9* is expressed in the genital ridge of both XX and XY embryos (Morais da Silva *et al*. 1996). In XY embryos, its expression increases on ED 11.5, just after the *Sry* peak, remaining high throughout testicular development but in the female genital ridge, the expression of *Sox9* is down-regulated (Kent *et al*. 1996, Morais da Silva *et al*. 1996). *Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X, gene 1 (Dax1)* is expressed in both XX and XY gonads and the onset of *Dax1* expression occurs at the same time as that of *Sry* (Swain *et al*. 1996). *Dax1* has been postulated to function as an anti-testis factor during gonadal development: overexpression of *Dax1* in the presence of weakened *Sry* allele causes sex reversal of XY mice indicating an antagonizing effect on *Sry* and male development (Swain *et al*. 1998). However, it has also been shown that *Dax1* is essential for the maintenance of germinal epithelial integrity and gametogenesis in the adult mouse testis; lack of functioning *Dax1* results in male sterility (Yu *et al*. 1998). *Wingless-type MMTV integration site family, member 4 (WNT4*), a member of *WNT* gene family, also has a pivotal role in gonadal development. In the XY gonads of a

mouse, *Wnt4* expression is downregulated in the somatic cell lineages after the activation of *Sry* around ED 11.5 but the expression remains high in the ovary (Vainio *et al*. 1999). As *Wnt4* expression is lost in the testis in conjunction with male sex determination, *Wnt4* may be negatively regulated by the male sex-determining pathway (Vainio *et al*. 1999). Lack of a functional *Wnt4* gene is known to result in masculinization of XX mouse embryos (Vainio *et al*. 1999).

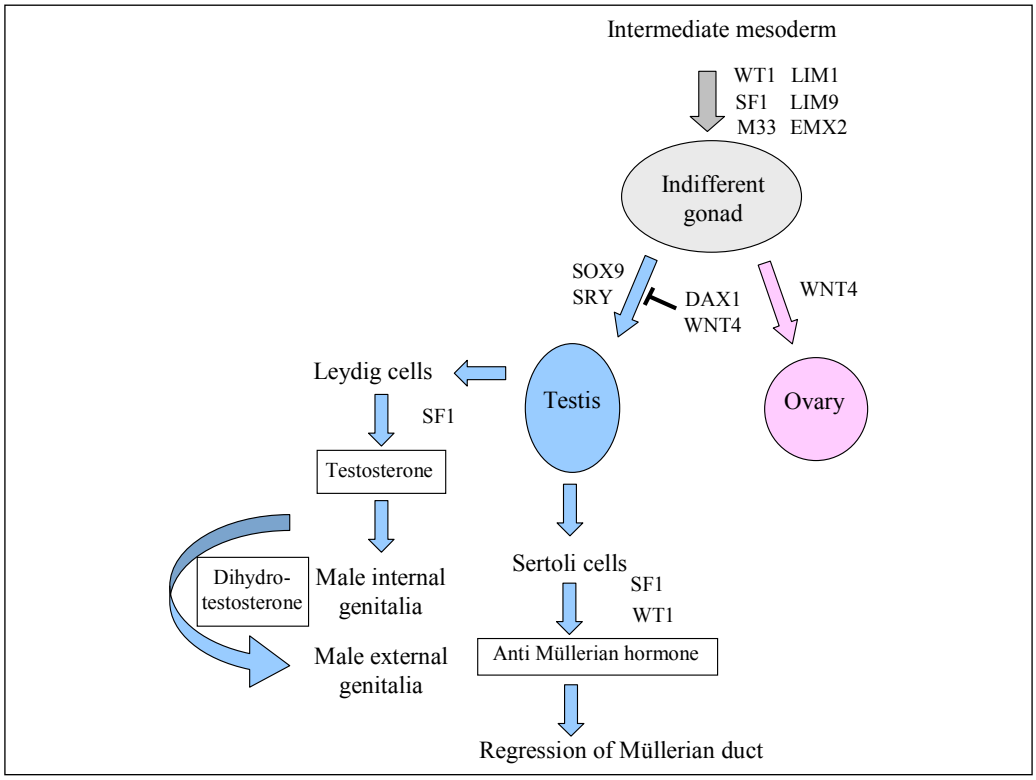

Figure 1. Schematic diagram illustrating the general pathways in gonadal differentiation. WT1, *Wilms' tumor suppressor 1;* SF1, steroidogenic factor 1; M33, modifier 3 protein; LIM1 and LIM9, *LIM homeobox* gene 1 and 9; EMX2, empty spiracles homeobox 2; WNT4, *wingless-related MMTV* integration *site 4*; DAX1, dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region of the X gene 1; SOX9, SRY-related high-mobility group (HMG) box 9; SRY, the sex-determining region of the Y chromosome.

2.2 TESTIS DEVELOPMENT

In the rat, the differentiation of testis becomes evident at ED 14: electron microscopic observations have revealed that at this time window seminiferous cords become visible within the gonadal ridge as a result of Sertoli cell and germ cell aggregation (Jost *et al*. 1981, Magre & Jost 1991). Testicular cords are composed of germ cells surrounded by Sertoli cells and peritubular myoid cells. In the rat, first Sertoli cells differentiate and polarize on ED 13 (Jost *et al*. 1981, Magre & Jost 1991). Developed Sertoli cells start to secrete anti-Müllerian

hormone (AMH) that causes the regression of Müllerian ducts in male embryos (Rey *et al*. 2003). The expression of *Amh* is switched off postnatally, coincident with the appearance of androgen sensitivity of the Sertoli cells (Munsterberg & Lovell-Badge 1991, Sharpe *et al*. 2003). Androgen action is mediated by androgen receptors (ARs). Studies with complete androgen receptor knock-out (ARKO) mice have demonstrated that androgens play a crucial role in regulating Sertoli cell proliferation in perinatal male mice; the number of Sertoli cells per testis was significantly reduced in the ARKO mice (Tan *et al*. 2005, Scott *et al*. 2007). In the fetal rat testis, ARs are expressed from ED 14.5 in interstitial cells and peritubular myoid cells but Sertoli cells do not express ARs until postnatal day (PND) 5 (You & Sar 1998). The proliferation of Sertoli cells increases progressively during prenatal life peaking on ED 20 in the male rat (Orth 1982). During the perinatal period, rat Sertoli cells start to secrete mature inhibin, a dimeric gonadal glycoprotein which selectively suppress follicle stimulating hormone (FSH) secretion from the anterior pituitary (Noguchi *et al*. 1997). At ED 20, the number of FSH receptors in Sertoli cells also starts to rise (Warren *et al*. 1984). This fits well with the fact that FSH is the main regulator of Sertoli cell function in postnatal testis. Studies with a Sertoli cell-depleted rat model have indicated that the size of the Sertoli cell population could define at least partly the production of the normal number of germ cells in the adult testis (Orth *et al*. 1988).

Interstitial cells differentiate from mesenchymal precursors (Byskov 1986). Fetal Leydig cells differentiate relatively late in the course of general testis formation. The signals triggering both the initial differentiation of fetal rat Leydig cells as well as the ultimate embryonic origin of these cells are still unknown (Habert *et al*. 2001). It has been suggested that the paracrine action of the Sertoli cells could play a role in the initiation of Leydig cell differentiation (Byskov 1986). The fundamental function of fetal Leydig cells is the production of testosterone (T) that is required for masculinization of the male urogenital system. In the rat testis, fetal Leydig cells appear on ED 14.5 and start to produce testosterone on ED 15 (Habert *et al*. 2001). In male rat T content reaches the maximal prenatal peak level on ED 18.5-19.5 (Tapanainen *et al*. 1984, El-Gehani *et al*. 1998) (Figure 2). This peak is physiologically associated to the increased number of fetal Leydig cells (Tapanainen *et al*. 1984, Huhtaniemi & Pelliniemi 1992). Fetal Leydig cells differ functionally and morphologically from the adult type of Leydig cells that replace the fetal population postnatally (Huhtaniemi &Pelliniemi 1992). Fetal Leydig cells are rich in lipid droplets and have more extensive cytoplasm than adult Leydig cells (Zirkin & Ewing 1987, Kerr & Knell 1988). Other morphological features that differentiate these two types of Leydig cells from each other are the endoplasmic reticulum and the surface area of the inner mitochondrial membrane that are more abundant in adult Leydig cells (Zirkin & Ewing 1987). It has been shown that in contrast to adult Leydig cells, fetal Leydig cells differentiate without luteinizing hormone (LH) stimulation (Saez 1994, Zhang *et al*. 2004): lack of LH or LH receptors does not disturb fetal Leydig cell function or fetal gonadal development (Kendall *et al*. 1995, O'Shaughnessy *et al*. 1998) but they do become stimulated in the presence of LH. *In vitro* studies have shown that in addition to LH/human chorionic gonadotropin (hCG), adrenocorticotrophic hormone (ACTH) can also stimulate fetal Leydig cell function during late gestation (O'Shaughnessy *et al*. 2003).

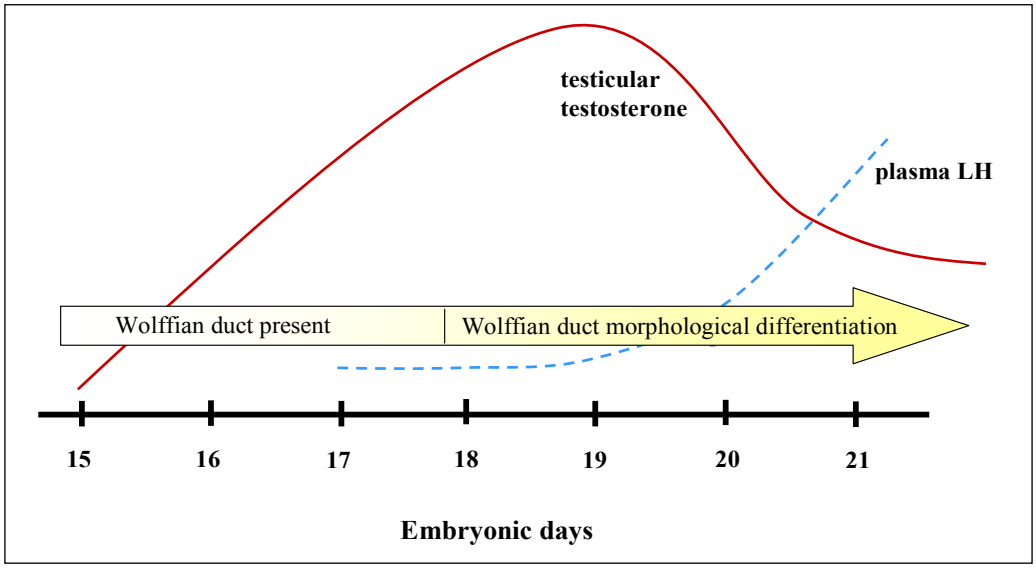

Figure 2. Schematic diagram illustrating the ontogeny of testicular testosterone and plasma luteinizing hormone (LH) production in fetal rat.

2.2.1 Desert hedgehog signalling in the fetal Leydig cell

The hedgehog (Hh) signalling pathway regulates numerous processes during embryonic development. It is involved for example in cell proliferation, limb morphogenesis and neural patterning (Goodrich & Scott 1998, Schneider *et al*. 1999, Ingham & McMahon 2001). In mammals, there are three Hh family members, Indian, Sonic and Desert that function as the ligands of the Hh signalling pathway (Ingham & McMahon 2001). In the testis, an extracellular protein Desert hedgehog (Dhh) produced and secreted by Sertoli cells (Bitgood & McMahon 1995) is an essential positive regulator in Leydig cell differentiation (Yao *et al*. 2002). It is expressed shortly after the onset of testis determining factor *Sry* (Bitgood *et al*. 1996). Dhh binds to the receptor Patched 1 (Ptch1) that is expressed in the fetal Leydig cells and in the peritubular myoid cells (Bitgood *et al*. 1996, Clark *et al*. 2000). Studies with homozygous Dhh-null mice have demonstrated that male embryos suffered from small testis size on ED 18.5 and infertility in adulthood, whereas females showed no apparent phenotype (Bitgood *et al*. 1996). Although *Ptch1* is expressed throughout the cells of the interstitium in fetal XY mouse, the disruption of *Dhh/Ptch1* signalling in Dhh-/- mice only seems to affect the differentiation of fetal Leydig cells; no effects have been reported on mesonephric cell migration or on the establishment of the interstitial cell population (Yao *et al*. 2002). In the adult mouse testis mRNAs of *Ptch2*, another patched receptor identified to date, are expressed in the spermatogonia, spermatocytes and in round spermatids (Szczepny *et al*. 2006).

In the absence of ligand (Figure 3), the Hh signalling pathway is inactive and the 12-transmembrane protein receptor Ptch represses the action of Smoothened (Smo), a seven-transmembrane protein that mediates the Hh signal (Kalderon 2000). Transcription

factor Gli (*glioma-associated oncogene*) is then prevented from entering the nucleus through interactions with the cytoplasmic complex that includes serine/threonine kinase Fused and Suppressor of fused (Sufu) (Pasca di Magliano &Hebrok 2003). Thus, the expression of target genes is inhibited. The hedgehog signalling pathway culminates in the activation of the transcription factors of the *Gli* family; there are three different Gli proteins in vertebrates and all of them have been implicated in mediating the activities of the various Hh proteins (Ruiz i Altaba *et al*. 2002). Hh signalling pathway is activated (Figure 3) when Hh binds to Patch. Ligand binding results in de-repression of *Smo* and activates a signalling cascade that includes upregulation of the *Gli*. Gli is translocated to the nucleus in which it modifies the expression of target genes (Pasca di Magliano & Hebrok 2003).

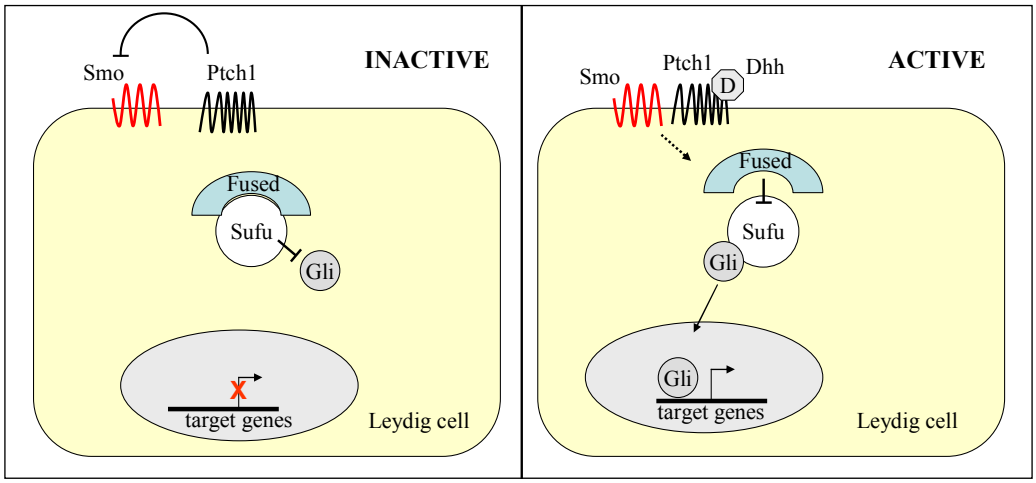

Figure 3. Desert hedgehog signalling pathway in Leydig cells. Dhh, desert hedgehog; Ptch1, patched 1; Smo, smoothened; Sufu, suppressor of fused (modified from Pasca di Magliano & Hebrok 2003).

In addition to the transcription factors of the Gli family, there are a number of other factors that contribute to the development of the gonads. *Steroidogenic factor 1* (*Sf1*) is known to be essential not only for the development of gonadal but also adrenal precursors (Luo *et al*. 1995). It is involved in the regulation of *insulin-like factor 3* (*Insl3*) transcription. INSL3 is a Leydig cell derived peptide hormone that is responsible for proper transabdominal descent of testis by regulating the growth and differentiation of gubernaculum (Nef & Parada 1999, Zimmermann *et al*. 1999). *GATA binding protein 4* (*Gata4*) plays a role in the development of Leydig cells (Bielinska *et al*. 2007) and Sertoli cells (Tevosian *et al*. 2002). It is also expressed in fetal adrenocortical cells (Kiiveri *et al*. 2002) in which it regulates adrenocortical gene regulation in association with *Sf1* (Tremblay & Viger 2003).

2.3 DEVELOPMENT OF MAMMALIAN ADRENAL GLANDS

The adrenal gland is composed of two distinct structures; the cortex and the medulla that differ in their embryological origin and function. The cells in the adrenal cortex originate

from the intermediate mesoderm and are a part of the adrenogonadal primordium within the urogenital ridge (Morohashi 1997). Adrenal medullary cells are derived from the embryonic neural crest (Morohashi 1997). The functional difference comes from their ability to synthesize different types of hormones: steroid hormones are synthesized in the adrenal cortex, whereas chromaffin cells in the medulla secrete catecholamines and several neuropeptides.

Several regulatory genes *[e.g. Wilms'tumor suppressor gene 1 (Wt1), Sf1 and Wnt4],* expressed in an exact spatio-temporal fashion, have been shown to play a crucial role in the differentiation of the adrenogonaldal primordium (Morohashi 1997, Bielinska *et al*. 2006, Kempna & Fluck 2008) (Figure 4). At the beginning of adrenal organogenesis, sympatho-adrenal cells migrate from the neural crest to the primordium (Anderson 1993). These cells give rise to medullary chromaffin cells in the middle of the gland. It has been demonstrated that mutations in the regulatory genes expressed in the adrenogonadal primordium can disturb not only the development of the adrenals but also the gonads due to the close relationship between these two lineages (Morohashi 1997, Keegan & Hammer 2002). Transgenic and knock-out mice models have provided new information about the organogenesis of the adrenal gland. However, the adrenal phenotypes induced by gene manipulation in mice may differ from those in humans (Else & Hammer 2005).

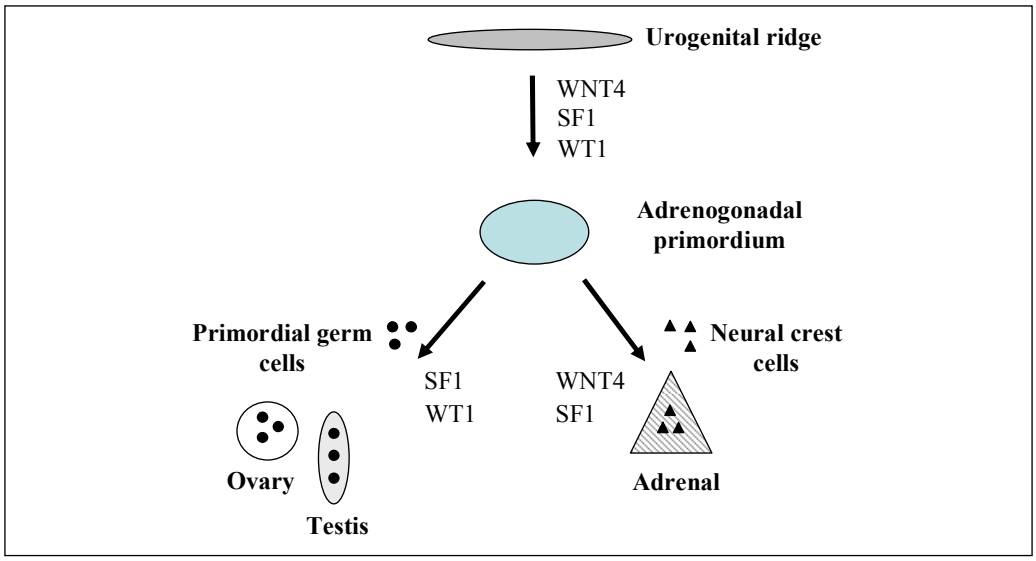

Figure 4. Development of the adrenal gland and gonads from the urogenital ridge. WNT4, *wingless-related MMTV* integration *site 4*; SF1, steroidogenic factor 1; WT1, *Wilms' tumor suppressor 1 (mo*dified form Bielinska *et al.* 2006).

In most mammals, the adrenal cortex is divided into three morphologically and functionally distinguishable layers: the *zona glomerulosa* which is located immediately below the capsule; the *zona fasciculata*, which is located in the middle; and the *zona reticularis*, which is the innermost zone next to the medulla (Kempna & Fluck 2008) (Figure 5). The *zona glomerulosa* produces mineralocorticoids whereas glucocorticoids and androgen

precursors are synthesized in the *zona fasciculata* and *zona reticularis*. However, in mouse and rat adrenals, no functionally distinct *zona reticularis* exists and they do not produce androgens in the adrenal cortex because of the tissue-specific lack of cytochrome P450 17α-hydroxylase /17,20-lyase (P450c17) required for androgen synthesis (Perkins & Payne 1988, Le Goascogne *et al*. 1991). The *zona intermedia*, a less prominent layer between the *zona glomerulosa* and *zona fasciculata* has also been detected in the adrenal cortex (Mitani *et al*. 1994). This undifferentiated zone lacks the ability to produce either glucocorticoids or mineralocorticoids but it has been proposed to contain adrenocortical stem cells in adult (Mitani *et al*. 1994). However, it has been found to be technically difficult to detect such undifferentiated cortical cells in fetal adrenals, because no specific markers are known for these cells (Mitani *et al*. 1999). The differentiation of the adrenocortical zones is believed to be due to differential temporal gene expression during fetal development and postnatal growth (Vinson 2003, Hammer *et al*. 2005).

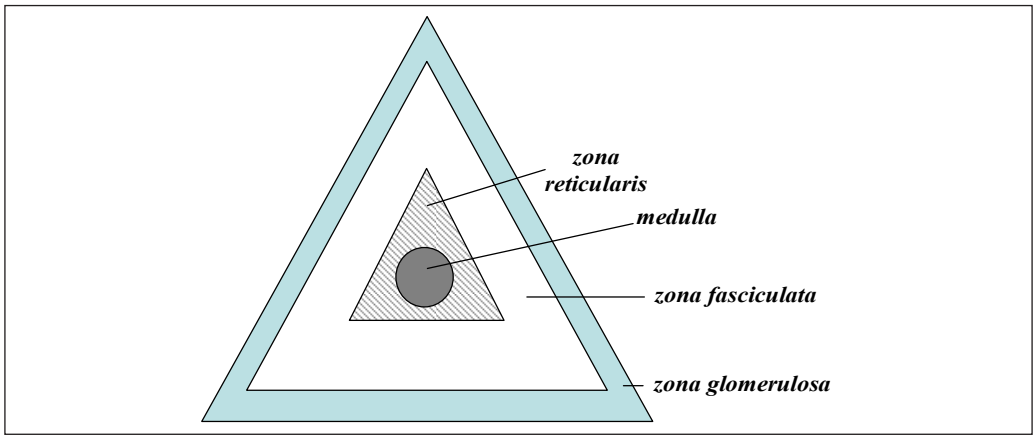

Figure 5. Schematic diagram of the adrenal gland showing its three cortical layers and medulla.

In rat, the separation of the cortex and medulla and the development of functional zonation in the adrenal cortex begins on ED 20 (Mitani *et al*. 1999). At this time point, most of the DNA-synthesizing cells, detected by 5-bromo-2-deoxyuridine (BrdU) and tyrosine hydroxylase labelling, are present in the outer portion of the adrenal gland as in the adult (Mitani *et al*. 1999). Immunohistochemical studies on fetal rat adrenals have revealed that steroidogenic enzymes cytochrome P450 cholesterol side chain cleavage (P450scc), cytochrome P450 21-hydroxylase (P450c21) and cytochrome P450 11β-hydroxylase (P450c11) are already expressed throughout the adrenal gland on ED 16 (Wotus *et al*. 1998, Mitani *et al*. 1999) although no cortical zonation is recognizable. The early expression of steroidogenic enzymes fits well with the observation that measurable corticosterone levels have been detected in fetal rat plasma from ED 16 onwards (Boudouresque *et al*. 1988). In rat, corticosterone production reaches the prenatal peak level on ED 19 (Boudouresque *et al*. 1988) (Figure 6). This prenatal peak in glucocorticoid production is essential for preparing the fetus for birth (Liggins 1994). It has also been shown that small amounts of adrenal aldosterone synthase

cytochrome P450 (P450aldo) are found for the first time on ED 16 (Wotus *et al*. 1998). By ED 20, the distribution pattern of P450aldo and P450c11 is changed; P450aldo-positive cells are localized in the outermost portion of the cortex and P450c11-positive cells inside of the P450aldo-positive cell layer indicating the true beginning of the functional zonation of the adrenal cortex (Mitani *et al*. 1999). Final adrenal zonation is established after birth (Mitani *et al*. 1999).

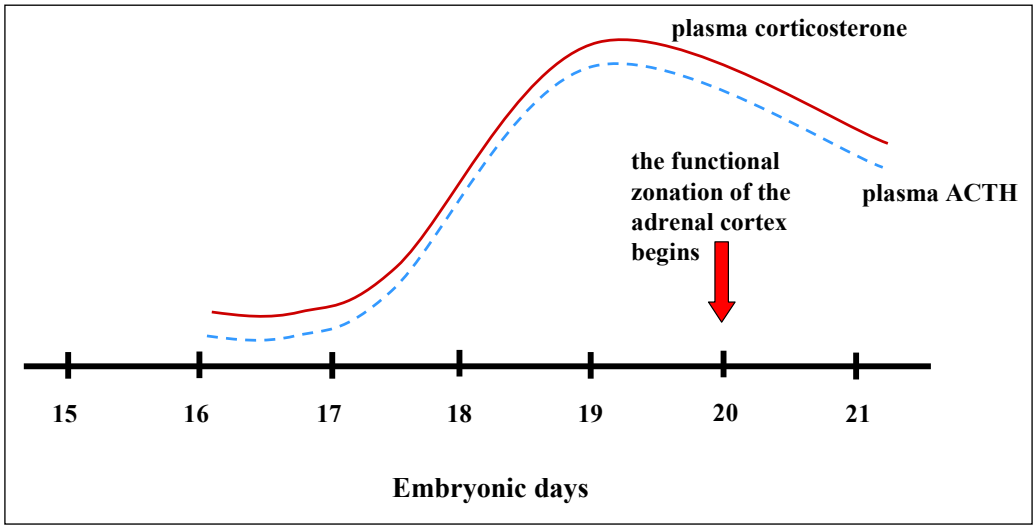

Figure 6. Schematic diagram illustrating the ontogeny of plasma corticosterone and adrenocorticotrophic hormone (ACTH) production in fetal rat.

2.4 MATURATION OF HYPOTHALAMUS-PITUITARY-ADRENAL/GONADAL (HPA/HPG) FUNCTION

ACTH, LH and FSH secreted from the anterior pituitary gland, stimulate adrenocortical and gonadal steroid producing cells to synthesize and secrete corticosteroids and sex steroids (androgens and estrogens) (Figure 7). The system by which these hormones are regulated is complex and involves feedback mechanisms from the hypothalamus, pituitary and adrenal cortex/gonads. Pulsatile secretion of corticotropin releasing hormone (CRH) and gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the secretion of corticotropin and gonadotropins (FSH and LH) from the pituitary gland, which in turn affects steroid secretion. Corticosteroids and sex steroids in turn negatively affect the synthesis and secretion of pituitary and hypothalamic hormones.

The mRNA levels of CRH can first be detected on ED 17 in rat in the parvocellular portion of the paraventricular nucleus (Baram & Lerner 1991). The expression levels of the CRH gene increases in the late fetal period and decreases around birth (Baram & Lerner 1991). Low levels of GnRH, the other hypothalamic hormone involved in the development of hypothalamus-pituitary-adrenal/gonadal axes (HPA and HPG axes, respectively), can be found already on ED 12 in the fetal rat brain and the concentrations of GnHR start to increase by the end of

gestation (Aubert *et al*. 1985). In fetal rat, CRH and GnRH begin to play a physiological role in the regulation of ACTH and gonadotropins, respectively, as early as on ED 17 (Salisbury *et al*. 1982, Boudouresque *et al*. 1988). Plasma ACTH levels become detectable on ED 16 (Boudouresque *et al*. 1988) and LH on ED 17.5 (Habert & Picon 1982, El-Gehani *et al*. 1998) in the rat. Both ACTH and LH levels increase thereafter reaching the prenatal peak value on ED 19 (Figure 6) and ED 21.5 (Figure 2), respectively (Boudouresque *et al*. 1988, El-Gehani *et al*. 1998). As a result of trophic hormone secretion, the steroidogenic activity of adrenocortical cells and testicular Leydig cells increases remarkably. The negative feedback regulation between the pituitary and adrenal gland begins to operate between ED 16 and 17 in the rat (Grino *et al*. 1995). The fetal rat pituitary-testicular axis becomes functional between ED 19.5-20.5 in the rat (Pakarinen *et al*. 1994).

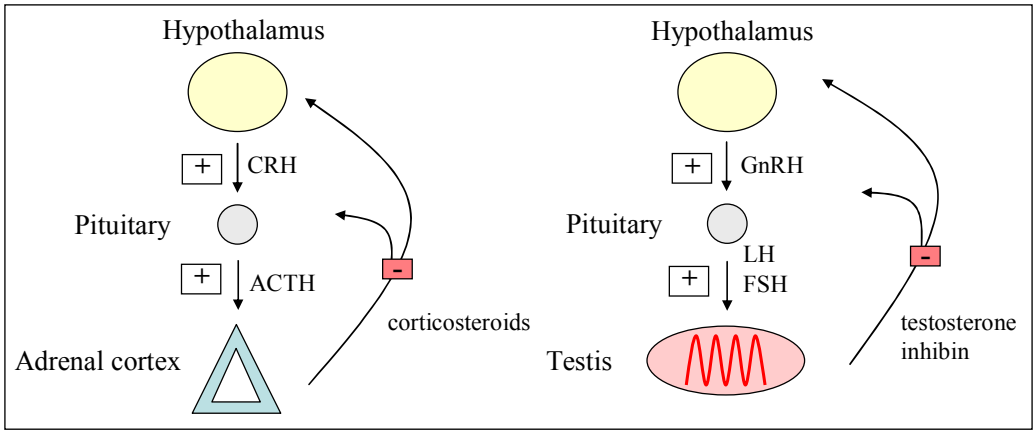

Figure 7. Schematic representation of the HPA and HPG axes. CRH, corticotropin releasing hormone; ACTH, adrenocorticotrophic hormone; GnRH, gonadotropin releasing hormone; LH luteinizing hormone; FSH, follicle stimulating hormone.

2.5 STEROIDOGENESIS

Steroid hormones can be categorized in five different groups according to the receptors to which they bind: mineralocorticoids, glucocorticoids, androgens, estrogens and progestagens. The biosynthesis of steroid hormones begins with the cleavage of the side chain of cholesterol to form pregnenolone (Figure 8). This reaction takes place in the matrix side of the inner mitochondrial membrane by the enzyme the P450scc (Simpson & Boyd 1966). To keep up steroidogenesis, the cell must provide a supply of substrate cholesterol to P450scc enzyme within the mitochondria for subsequent production of steroid hormones. The delivery of cholesterol to the inner mitochondrial membrane by Steroidogenic Acute Regulatory protein (StAR) is the rate-limiting step in the steroidogenic pathway (Stocco 2000). StAR protein is acutely regulated and the expression of protein is critically dependent on trophic hormone stimulation (*e.g.* LH and ACTH). This makes it susceptible to environmental toxicants: several xenobiotics [*e.g*. 4-*tert*-octylphenyl and pesticides Lindane (1,2,3,4,5,6-hexachlorocyclohexane) and glyphosate Roundup (2-(phosphonomethylamino)

acetic acid)] have been reported to disrupt steroidogenesis by inhibiting the expression of the StAR protein (Walsh & Stocco 2000, Walsh *et al*. 2000, Myllymaki *et al*. 2005b). Mutations in the StAR gene is known to cause the disease lipoid congenital adrenal hyperplasia (lipoid CAH) (Lin *et al*. 1995, Bose *et al*. 1996). Lipoid CAH is an autosomal recessive lethal condition in which cholesterol and cholesterol esters accumulate and the newborn child is unable to synthesize a sufficient amount of steroids. StAR knockout mice display a phenotype that is very similar to lipoid CAH in humans providing a good model in which to study the mechanism of StAR protein's essential contribution to steroidogenesis and endocrine development (Caron *et al*. 1997).

Pregnenolone, the precursor of all steroid hormones, diffuses across the mitochondrial membranes to the cytoplasm in which it is further metabolized by the enzymes associated with the smooth endoplasmic reticulum. 3β-hydroxysteroid dehydrogenase (3β-HSD), one of the few non-CYP450 enzymes involved in steroidogenesis, converts Δ^5-3β-hydroxysteroid to Δ^4-3-ketosteroids. The enzyme 3β-HSD is widely expressed in steroidogenic and non-steroidogenic tissues such as the testis, prostate, skin and brain (Simard *et al*. 2005). In the rat, four isozymes of 3β-HSD have been characterized showing differential and tissue-specific expression (Simard *et al*. 1993).

The cytochrome P450 17α-hydroxylase/17-20 lyase (P450c17) has two distinct catalytic activities. I) It mediates 17α-hydroxylation of pregnenolone or progesterone (P4) resulting in the production of hydroxypregnenolone or hydroxyprogesterone. II) The enzyme also mediates the cleavage of the C17,20 bond of these compounds forming a weak androgen, dehydroepiandrosterone (DHEA) or androstenedione. The C17,20-lyase is highly expressed in the gonads and it is essential for directing the biosynthesis of steroids towards the sex hormones (Miller *et al*. 1997). Androstenedione is converted to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). Several types of 17β-HSD have been characterized. 17β-HSD type 3 is primarily expressed in the testis (Andersson 1995). Androgens (androstenedione and T) can be irreversibly transformed into estrogens by cytochrome P450 aromatase (P450arom). In the mammalian testis, low levels of P450arom are expressed not only in Leydig cells, but also in Sertoli cells and germ cells (Carreau *et al*. 2002). Dihydrotestosterone (DHT), the most potent endogenous androgen, is formed from testosterone by 5α-reductase. There are two isozymes of 5α-reductase that have differential biochemical and molecular features (Imperato-McGinley & Zhu 2002): type 1 has a neutral to basic pH optimum and it is mainly expressed in the peripheral tissues (*e.g*. skin, kidneys, intestine and liver) and type 2 that has an acidic pH optimum and is predominantly expressed in the prostate, epididymis and seminal vesicles (Jenkins *et al*. 1992, Normington & Russell 1992, Imperato-McGinley & Zhu 2002). DHT induces the differentiation of male accessory sex organs (Wilson 1978). Thus, 5α-reductase is a critical determinant of androgen activity in these tissues.

Rats and mice do not express P450c17 in the adrenal cortex as mentioned earlier (Perkins & Payne 1988, Le Goascogne *et al*. 1991). They use corticosterone as a glucocorticoid instead of cortisol. In the adrenal cortex, the enzyme P450c21 catalyzes the 21-hydroxylation of

both glucocorticoids and mineralocorticoids. In the *zona fasciculata* and *zona reticularis*, mitochondrial cytochrome P450c11 mediates the conversion of 11-deoxycorticosterone to corticosterone (Ogishima *et al*. 1992, Ho & Vinson 1993). The synthesis of cortisol, the main glucocorticoid produced in humans, also involves the catalyzing action of P450c11. Corticosterone can be further metabolized to aldosterone which is the most potent steroid regulating electrolyte balance. This reaction is catalyzed by mitochondrial P450aldo in the *zona glomerulosa* (Ogishima *et al*. 1992).

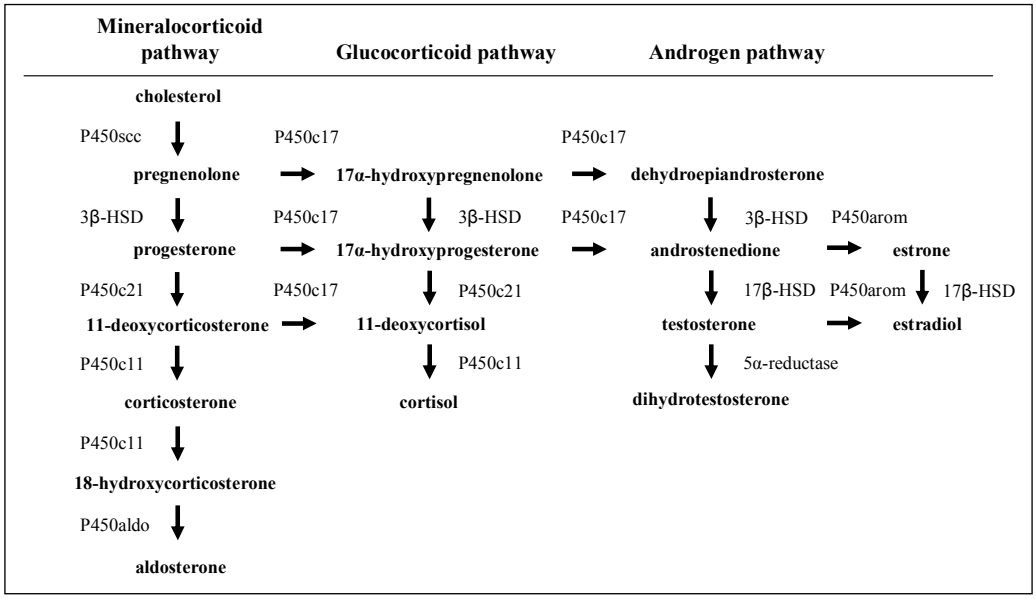

Figure 8. Major pathways in steroid biosynthesis. P450scc, cytochrome P450 cholesterol side chain cleavage; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c17, cytochrome P450 17α-hydroxylase /17,20-lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; P450c21, *cytochrome P450* 21-hydroxylase; P450c11, cytochrome P450 11β-hydroxylase; P450arom, cytochrome P450 aromatase; P450aldo, aldosterone synthase cytochrome P450.

2.6 TESTICULAR DYSGENESIS SYNDROME (TDS)

There is considerable epidemiological evidence for the increasing incidence of congenital disorders of the male reproductive system: these health disorders include among others increasing incidence of hypospadias, cryptorchidism (Matlai & Beral 1985), testicular cancer (Adami *et al*. 1994, Moller 1998) and declining semen quality (Carlsen *et al*. 1992). In 2001, Skakkebæk and colleagues (Skakkebaek *et al*. 2001) hypothesized that all these clinical abnormalities observed in the male reproductive system are symptoms of one underlying entity "testicular dysgenesis syndrome" (TDS), and that the ultimate cause for them is of fetal origin. TDS may be caused by environmental factors or genetic defects (*e.g.* 45X/46XY and androgen insensitivity (Virtanen *et al*. 2005), or combination of both (Figure 9). TDS may lead secondly to malfunctioning of testicular Leydig and/or Sertoli cells during male sexual differentiation. This in turn may cause androgen insufficiency, weakened INSL3

production and/or impaired germ cell differentiation and eventually, this may lead to clinical manifestations of TDS (Boisen *et al*. 2001, Skakkebaek *et al*. 2001, Joensen *et al*. 2008).

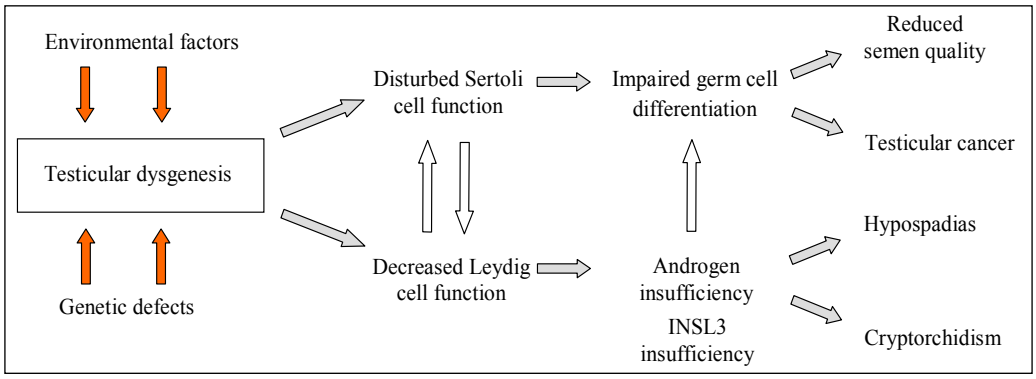

Figure 9. Schematic diagram illustrating the general pathways in which the disorders that comprise testicular dysgenesis syndrome may arise (modified form Skakkebæk *et al*. 2001).

2.7 ENDOCRINE DISRUPTERS

Environmental contaminants capable of interfering with reproduction and other endocrine regulated functions have received increased attention in the context of wildlife and human health (Guillette & Gunderson 2001, Joffe 2001, Norgil Damgaard *et al*. 2002, Hotchkiss *et al*. 2008). The term endocrine disrupter is defined "as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". This definition was recommended by the International Programme on Chemical Safety (http://whqlibdoc.who.int/hq/2002/a76784_ch1.pdf). Currently there is a lot of data concerning the adverse health effects of endocrine disrupters in laboratory animals; several screening and testing assays have been developed to identify endocrine disrupting chemicals (Gray *et al*. 2002).

Androgens are critical determinants of mammalian male phenotype (Wilson 1978) and exposure to chemicals with antiandrogenic properties during the critical developmental window (*i.e*. sexual differentiation) can have permanent effects on the male reproductive tract (Welsh *et al*. 2008). Recent data have shown that mixtures of endocrine disrupting chemicals with diverse mechanisms of toxicity can display cumulative, dose additive or synergistic effects on reproductive tract development in male experimental animals (Hass *et al*. 2007, Christiansen *et al*. 2008, Kortenkamp 2008, Rider *et al*. 2008). To assess the risk of endocrine disrupting chemicals to human health and wildlife, only associations can be made. Therefore, more focused research about the long-term health effects associated with exposure to endocrine disrupters during early life is needed. In Figure 10 the chemical structures of selected chemicals with antiandrogenic properties are presented.

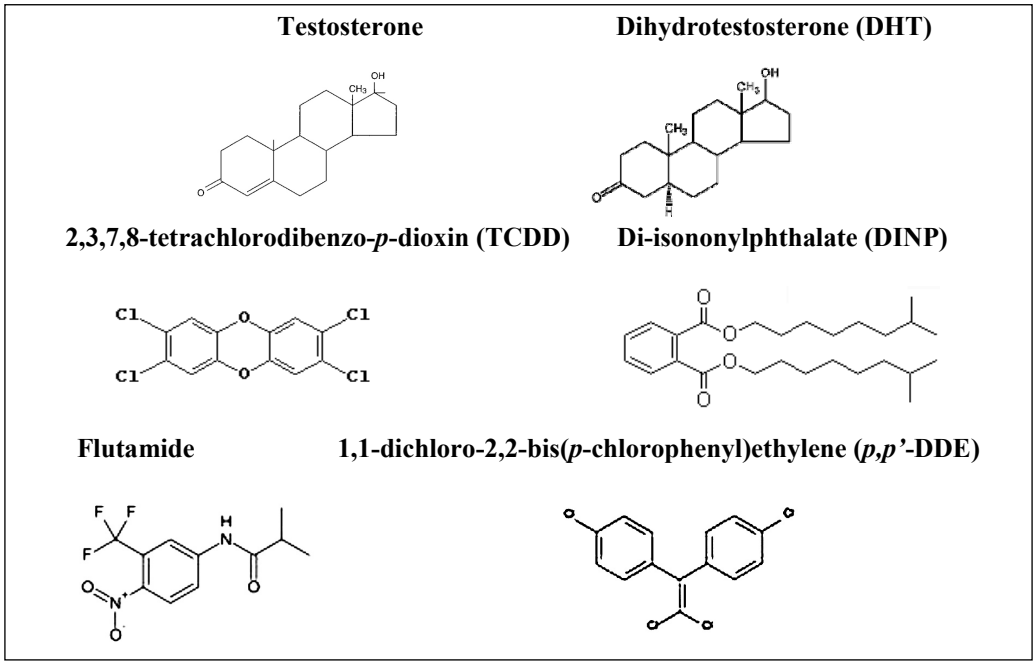

Figure 10. Chemical structures of selected antiandrogens and endogenous androgens.

2.7.1 Diethylstilbestrol

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen (Figure 11) that acts as a transplacental toxicant causing a wide variety of physiological effects in the reproductive tract of female and male offspring when administered during pregnancy to humans and laboratory animals (Newbold 2004). It was first synthesised in 1938. From the 1940s to 1960s, DES was used to treat patients with the threat of miscarriage and late pregnancy complications such as toxemia, stillbirth and premature delivery (Palmlund 1996). It became evident that DES-treatment during pregnancy had no therapeutic value (Dieckmann *et al*. 1953) and numerous studies describing the detrimental effects of DES on the reproductive health of male and female offspring were published: prenatal exposure to DES has been reported to cause cervicovaginal clear-cell adenocarcinoma, adenosis and structural malformations in the reproductive tract of female offspring (Herbst *et al*. 1975, Herbst 1976). In males, DES-induced effects include among others cryptorchidism, hypotrophic testis, epididymal cysts and microphallus (Gill *et al*. 1976, Gill *et al*. 1979). The clinical use of DES was banned in 1971 by the U.S. Food and Drug Administration (Giusti *et al*. 1995). Today the mechanisms involved in DES-induced reproductive disorders are well characterised and DES is widely used as an estrogenic model compound in reproductive toxicity studies.

DES has a low binding capacity to alpha fetoprotein (AFP) and sex hormone binding globulins (SHBG) (Sheehan & Young 1979, Savu *et al*. 1981, Milligan *et al*. 1998, You *et al*. 1998), proteins that normally protect the fetus from maternal steroid hormones. The ability of DES to cross the placental barrier may severely affect the developing fetus. DES has a high affinity to estrogen receptor (ER) binding sites (Kuiper *et al*. 1997). Experimental studies suggest that

reproductive abnormalities resulting from developmental DES exposure are a consequence of estrogen receptor alpha (ERα) activation in the tissues (Couse *et al*. 2001, Couse & Korach 2004, Cederroth *et al*. 2007). However, there is strong evidence that disturbance of the normal androgen-estrogen balance in the fetal environment rather than estrogenic effect alone contributes to DES-induced reproductive tract abnormalities in males (McKinnell *et al*. 2001, Williams *et al*. 2001, Rivas *et al*. 2002a). DES interferes with steroid hormone synthesis; in utero exposure to DES (100 and 500 µg/kg of maternal body weight) has been shown to decrease the expression levels of StAR and P450c17 in the fetal murine testis (Majdic *et al*. 1996, Saunders *et al*. 1997, Guyot *et al*. 2004). Declined mRNA and protein expression levels of SF1 have also been reported in DES-exposed testis (Saunders *et al*. 1997, Ikeda *et al*. 2008) although there are contradictions in the data. Unaltered or even increased levels of fetal testicular SF1 have also been described in animal experiments (Visser *et al*. 1998, Emmen *et al*. 2000, Guyot *et al*. 2004). This discrepancy between the expression levels of SF1 may result from differences in the prenatal exposure window. Reduced testicular Insl3 mRNA expression levels and maldeveloped gubernaculum ligaments have been detected in DES-exposed mouse fetuses; this observation clarifies the mechanisms of DES-induced cryptorchidism (Emmen *et al*. 2000). DES-induced abnormalities in the male reproductive tract and altered testicular steroidogenesis are not only a matter of high dose exposure. *In utero* exposure to low doses of DES (0.5 and 1.5 µg/kg) by daily subcutaneous injections on ED 7-21 resulted in decreased endocrine function in the testis of Sprague-Dawley rats at the age of six weeks (Yamamoto *et al*. 2005). Several studies have shown that developmental exposure to DES alters the expression of the *Hox* and *Wnt* family genes in the reproductive tract of female offspring (Miller *et al*. 1998, Block *et al*. 2000, Suzuki *et al*. 2007). These genes are critical for normal female reproductive tract development (Sassoon 1999, Taylor 2000). It has been proposed that perinatal DES exposure could alter the methylation status of estrogen-responsive genes (Li *et al*. 1997, Li *et al*. 2003). DNA methylation is known to affect transcriptional regulation. An altered methylation pattern contributes to tumorigenesis (Robertson & Jones 2000), which has been detected as an adverse consequence of DES exposure.

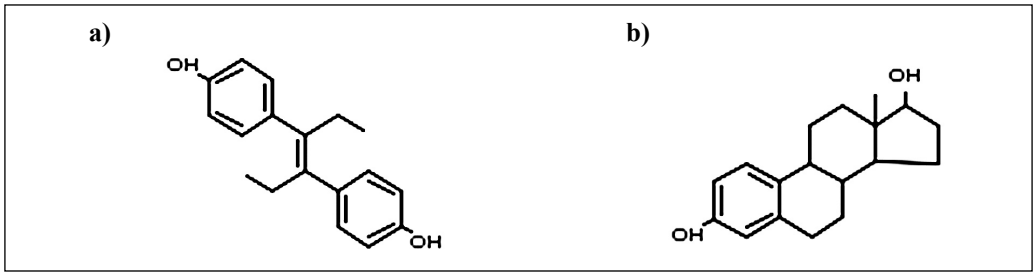

Figure 11. Chemical structures of a) diethylstilbestrol (DES) and b) 17β-estradiol.

2.7.2 Flutamide

Flutamide (4'-nitro-3'-trifluoromethyl-isobutyranilide) is a non-steroidal antiandrogenic drug that has been used to treat androgen dependent prostate cancer (Delaere & Van Thillo

1991, Klotz 2008). Flutamide and its active metabolite hydroxyflutamide function as an AR antagonist by competing with endogenous T and DHT for binding to the receptor. Several studies have described reduced anogenital distance (AGD), retained nipples, decreased accessory sex organ weights, cryptorchidism, hypospadias and hypospermatogenesis among the effects observed in male experimental animals exposed *in utero* and lactationally to flutamide (Imperato-McGinley *et al*. 1992, McIntyre *et al*. 2001, Miyata *et al*. 2002, Goto *et al*. 2004). Due to its well-known antiandrogenic properties, flutamide is used as a positive control in many bioassays (*e.g*. Hershberger and enhanced OECD Test Guideline no. 407) to screen chemicals with androgenic or antiandrogenic characteristics (Toyoda *et al*. 2000, Yamada *et al*. 2000).

It has been shown that perinatal exposure to flutamide affects androgen dependent organs in male offspring already at a rather low exposure level; decreased AGD has been observed at a dose of 2.5 mg/kg (Miyata *et al*. 2002). However, the amount and severity of flutamide-induced anomalies increases with the dose (McIntyre *et al*. 2001, Miyata *et al*. 2002). *In utero* exposure to flutamide induces testicular Leydig cell hyperplasia in prenatal and adult rat (Mylchreest *et al*. 1999, Mylchreest *et al*. 2002). Chronic apoptotic germ cell death associated with a long-term increase in the expression and activation of effector caspases -3 and -6 has also been detected in adult rat testis treated *in utero* with flutamide (Omezzine *et al*. 2003). The degree of morphological damage in the testis of prenatally flutamide–treated males is related to testis position, testicular weight and androgen levels (Kassim *et al*. 1997).

2.7.3 Di-isononylphthalate

Di-isononylphthalate (DINP), like other phthalate esters (PEs), is a synthetic chemical, primarily used as a plasticizer in the production of polymeric materials; it increases the flexibility and workability of polymers (Kavlock *et al*. 2002). Due to its widespread production and use, PEs have been studied quite extensively. DINP is a complex substance that is produced by a condensation reaction between phthalic anhydride and isononyl alcohol (http://ecb.jrc.it/Documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf). It is widely used in children's soft plastic teethers and toys, sealants, flooring tiles and wood veneer (Kavlock *et al*. 2002). DINP is not chemically bound to the polyvinyl chloride (PVC) plastics. Due to its physicochemical properties and common use in children's toys, the potential health risk of DINP exposure to infants has been of special concern (Babich *et al*. 2004).

Some PEs are endocrine-active chemicals by acting as antiandrogens without interacting directly with AR (Mylchreest *et al*. 1999, Parks *et al*. 2000). *In utero* exposure to PEs inhibit T production and *Insl3* expression by fetal Leydig cells in rat (Parks *et al*. 2000, Barlow *et al*. 2003, Borch *et al*. 2004, Wilson *et al*. 2004). Decreased T production by PEs during sexual differentiation may lead to disorders in the reproductive tract of male offspring. Perinatal exposure to DINP at 750 mg/kg/day has been reported to cause small and atrophic testis, epididymal agenesis and female-like nipples in the male rat (Gray *et al*. 2000). In addition to disrupted Leydig cell function, developmental exposure to PEs target Sertoli cells and

gonocytes; for example decreased levels of transcription factor *Dax*-1 and inhibin alpha have been described in male rat fetuses treated with dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) or benzyl butyl phthalate (BBP) (Liu *et al*. 2005). Disturbed interactions between Sertoli cells and gonocytes may lead to tubular malformations, multinucleated gonocytes or even reduced fertility (Liu *et al*. 2005, Ferrara *et al*. 2006). However, it has been shown that DINP has a lower antiandrogenic potency than some other phthalates, such as DEHP or BBP (Gray *et al*. 2000, Borch *et al*. 2004). DBP at a dose of 500 mg/kg of maternal body weight on EDs 12-19 has also been shown to decrease testosterone production and the expression levels of genes involved in testicular steroidogenesis of the fetal male rat (Barlow *et al*. 2003). PEs are rapidly hydrolyzed to the monoesters in the gastrointestinal tract. In repeated dose studies in which adult rats were exposed daily for five consecutive days of 50, 150 or 500 mg/kg of DINP ca. 60 % of the administered dose was excreted at all doses and the half-life for elimination of absorbed phthalate was ca. 7 h (McKee *et al*. 2002). Monoesters can also cross the placental barrier (Fennell *et al*. 2004, Calafat *et al*. 2006). The reproductive toxicity of phthalates is considered to be a consequence of monoester activity (Li *et al*. 1998, Ema & Miyawaki 2001).

2.7.4 DDT and *p,p'*-DDE

1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) is the main metabolite of the synthetic pesticide 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane (DDT), of which large-scale industrial production started in 1943 (Turusov *et al*. 2002). DDT has been used for over 60 years to control agricultural and forest pests and to protect against malaria and other vector-borne diseases (Turusov *et al*. 2002, Rogan & Chen 2005). It has even been applied as a louse-control substance in humans (Rogan & Chen 2005). Non-metabolized DDT and *p,p'*-DDE are highly lipophilic and metabolically resistant (Morgan & Roan 1971). They bioaccumulate in body fat with age and biomagnify along the food chain. In plasma, the half-life of *p,p'*-DDE has been estimated to be approximately 8 years (Axmon & Rignell-Hydbom 2006). The use of DDT was banned in developed countries in the 1970s mainly due to its harmful environmental effects (Rogan & Chen 2005). Eggshell thinning and breeding failures was observed in raptorial and fish-eating birds with DDT and its metabolites (Hickey & Anderson 1968, Elliott *et al*. 1988). Another example of the putative endocrine disrupting effects of DDT is alligators in Lake Apopka which exhibited abnormal levels of sex steroid hormones and reproductive defects (Guillette *et al*. 1994). However, due to its low cost DDT is still used for malarial control in some developing countries although resistance of insects to DDT has occurred worldwide (Sadasivaiah *et al*. 2007). The potential adverse effects of DDT and *p,p'*-DDE on human health have been widely studied. It has been suspected that exposure to DDT could have carcinogenic effects in humans; positive associations with certain cancers *e.g*. pancreatic cancer and breast cancer have been suggested (Garabrant *et al*. 1992, Wolff *et al*. 1993, Beard *et al*. 2003) but the data are controversial and inconclusive (Hunter *et al*. 1997, Zheng *et al*. 1999, Cocco *et al*. 2000). Neurological effects and reproductive abnormalities have also been linked to DDT exposure but as with the carcinogenicity of the chemical, the results are equivocal (Longnecker *et al*. 1997).

In vivo and *in vitro* studies have demonstrated that *p,p'*-DDE inhibits androgen binding to AR and subsequent androgen-induced transcriptional activity (Kelce *et al*. 1995). However, its binding affinity to AR *in vitro* is approximately 1/10 that of hydroxyflutamide (Kelce *et al*. 1995). *In vivo* studies have shown that indicators of prenatal antiandrogen exposure, reduction of AGD and nipple retention, were much more affected in male rats treated *in utero* with flutamide 40 mg/kg of maternal body weight from ED 14 to ED 18 than in males treated with *p,p'*-DDE 100 mg/kg with the same dosing regimen. This suggests a notably weaker binding to AR with *p,p'*-DDE also *in vivo* (You *et al*. 1998). However, there are differences in the sensitivity of rat strains to the antiandrogenic effects of *p,p'*-DDE: Long Evans rat seems more sensitive to the *p,p'*-DDE than the Sprague-Dawley rat that is commonly used for reproductive toxicity studies (You *et al*. 1998). In addition to the well-known characteristics of *p,p'*-DDE as an AR antagonist it has been suggested that *p,p'*-DDE could also affect androgen-sensitive tissues by stimulating conversion of T to less active metabolites and thereby decrease circulating T levels (Freyberger *et al*. 2007).

Metabolites of DDT are also well-known for their cytotoxic effects on the adrenal cortex; particularly 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD; mitotane) that has been used as an important anticancer drug to treat adrenocortical carcinoma and Cushing's syndrome (Schteingart *et al*. 1982, Orth 1995, Gonzalez *et al*. 2007). Several studies indicate that mitochondrial P450c11 in the adrenal cortex is targeted by DDT metabolites (Jonsson *et al*. 1991, Jonsson *et al*. 1995, Lund & Lund 1995). Due to this enzyme specificity, corticosteroid synthesis is disrupted.

2.7.5 Dioxins and TCDD

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic dioxin congener. In general, the term "dioxin" refers to a heterogenous mixture of polyhalogenated (usually polychlorinated) dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs). Dioxins are the by-products of diverse industrial processes (*e.g.* paper bleaching and manufacturing of chlorinated chemicals) and incomplete burning. Dioxins are lipophilic and persistent chemicals showing resistance towards metabolism. They biomagnify along the food chain. The main route of exposure for humans is diet (Kulkarni *et al*. 2008). In Finland the principal source for dioxin exposure is fatty Baltic Sea fish (Kiviranta *et al*. 2001); the Baltic Sea is heavily polluted with persistent organic pollutants, such as PCBs and other hydrophobic organochlorine compounds (Mackenzie *et al*. 2004).

Both epidemiological studies and animal experiments have shown that dioxin exposure is associated with several adverse health effects including immuno- and neurotoxicity, body weight loss ("wasting syndrome" in animal models), developmental toxicity, reproductive defects and carcinogenicity (Peterson *et al*. 1993, Birnbaum 1994, Pohjanvirta & Tuomisto 1994, Pelclova *et al*. 2006, Mocarelli *et al*. 2008). However, it is well-known that in addition to the dose of exposure, factors such as age, sex, animal species or even animal strain strongly affect the toxic responses of TCDD. The largest interspecies difference in acute TCDD toxicity among laboratory mammals has been described between guinea pig and hamster.

The lethal dose 50 % (LD_{50}) value ranges from 1-2 µg/kg in guinea pigs (Schwetz *et al*. 1973, McConnell *et al*. 1978) to 3000-5000 µg/kg in hamsters (Olson *et al*. 1980, Henck *et al*. 1981). A considerable intraspecies difference in TCDD sensitivity has been reported between the rat strains Long-Evans *(Turku/AB)* and Han/Wistar *(Kuopio)*: Long-Evans is very sensitive to the toxicity of TCDD (LD_{50}: 18 µg/kg) whereas Han/Wistar shows a great resistance to TCDD being even 1000-fold more resistant (LD_{50}: >9600 µg/kg) than Long-Evans (Pohjanvirta *et al*. 1993, Unkila *et al*. 1994). The molecular basis for the differences between Han/Wistar and Long-Evans is associated with a point mutation in the AhR gene resulting in a truncated transactivation domain of the receptor and exceptional resistance to TCDD in Han/Wistar rat (Pohjanvirta *et al*. 1998, Pohjanvirta *et al*. 1999, Tuomisto *et al*. 1999).

Developmental and reproductive disorders induced by *in utero* and lactational exposure to TCDD have been considered as the most sensitive endpoints of TCDD toxicity (Peterson *et al*. 1993). Nevertheless, the sensitivity of the male rat reproductive system to TCDD is greatly influenced by the timing of exposure (Bjerke &Peterson 1994, Ohsako *et al*. 2002). In male experimental animals, exposure to TCDD during development has been shown to interfere with the androgen-dependent mechanism and result in the decrease of AGD, delay of testicular descent, reduction in the weight of accessory sex organs and in the suppression of epididymal sperm number (Mably *et al*. 1992a, Mably *et al*. 1992b, Gray *et al*. 1995, Ohsako *et al*. 2001, Simanainen *et al*. 2004b). Already a relatively low dose exposure can have severe consequences for the development of androgen dependent tissues. In a study by Ohsako and colleagues (Ohsako *et al*. 2001), TCDD 200 ng/kg of maternal body weight was sufficient to significantly decrease the weight of the ventral prostate of adult male Holzman rats. Reduction in AGD occurred at doses higher than 50 ng/kg of maternal body weight. It seems that not only the development of the ventral prostate but also AGD are much more sensitive endpoints to TCDD exposure than testicular and other internal genital organs or daily sperm production (Ohsako *et al*. 2001). In the same study, down-regulated testicular mRNA levels of *AR* and increased *5α-reductase type 2* was detected in pubertal Holzman rats exposed *in utero* to low doses (12.5, 50, 200 and 800 ng/kg) of TCDD without affecting serum gonadotropin or T levels. The decreased size of the ventral prostate induced by maternal TCDD-treatment is thought to be due to the decreased responsiveness of prostate to androgens without inhibition of androgen production (Ohsako *et al*. 2001). Other studies have also indicated that the effects of developmental TCDD exposure on the male reproductive tract are not accompanied by impaired androgenic status (Gray *et al*. 1995, Roman *et al*. 1995, Theobald *et al*. 2000).

The contribution of genetic factors to the reproductive toxicity of male rats has been studied in rat lines selectively bred from TCDD-resistant Han/Wistar *(Kuopio)* and TCDD-sensitive Long-Evans *(Turku/AB)*. It has been shown that the effects of TCDD on the male reproductive organs after adult TCDD-exposure are not modified by resistance alleles. However, resistance to TCDD-induced decrease in spermatogenesis appears to be increased by Han/Wistar-type of AhR. (Simanainen *et al*. 2004a)

2.7.5.1 AhR and the mechanism of action

Most of the toxic effects of TCDD are mediated through binding to a specific receptor, aryl hydrocarbon receptor (AhR) (Poland & Glover 1980, Fernandez-Salguero *et al*. 1997, Tuomisto *et al*. 1999). AhR acts as a multifunctional molecular switch regulating endo-and xenobiotic metabolism, cell growth and proliferation (Bock & Kohle 2006) but the physiologic function of the receptor is still largely unknown. In the cytoplasm, AhR is bound to two 90 kDa heat shock proteins (hsp90) that act as molecular chaperones and some other proteins (Figure 12) (Petrulis & Perdew 2002). Upon binding of TCDD, the receptor complex translocates into the nucleus, where AhR heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and releases hsp90 and the other proteins associated with it in the cytoplasm. The AhR/ARNT heterodimer binds to a xenobiotic response element (XRE) located upstream of the target genes and regulates the transcription of genes such as cytochrome P450 1A1 (CYP1A1), a xenobiotic metabolizing enzyme (Mimura & Fujii-Kuriyama 2003). The negative regulator of AhR, aryl hydrocarbon receptor repressor (AhRR), competes with AhR for dimerization with ARNT and for binding to the XRE sequence (Mimura *et al*. 1999) but it also functions as transcriptional repressor. It has been shown that mRNAs for AhRR are present in the testis of adult rat and there are no marked differences between Han/Wistar and Long-Evans rats, implying that AhRR is not the auxiliary contributing factor to the strain difference in TCDD sensitivity (Korkalainen *et al*. 2004). Both AhR and ARNT have also been detected in the adult male rat reproductive tract; proteins are present in the testis, epididymis, vas deference, seminal vesicle and prostate (Roman *et al*. 1998, Schultz *et al*. 2003).

It has been proposed that the mechanism of TCDD-induced disorders in the reproductive system of mammals could involve I) altered steroidogenesis (Baba *et al*. 2005, Myllymaki *et al*. 2005a, Mutoh *et al*. 2006), II) reduced expression of sex steroid and LH receptors (Tian *et al*. 1998, Ohsako *et al*. 2001, Fukuzawa *et al*. 2004) by promoting the ubiquitination and degradation of receptor protein (Ohtake *et al*. 2007), III) modulation of ER signalling by a co-regulatory-like function of activated AhR/ARNT (Ohtake *et al*. 2003) or IV) induction of cytochrome P450 1 (CYP1) family enzymes resulting in enhanced inactivation of steroid hormones (Badawi *et al*. 2000). It is likely that the adverse effects following prenatal TCDD exposure are a result of a combination of the mechanisms described above. Other possibilities are overlapping enhancer regions for ER and AhR (Krishnan *et al*. 1994) and competition on ARNT between AhR and ER (Brunnberg *et al*. 2003).

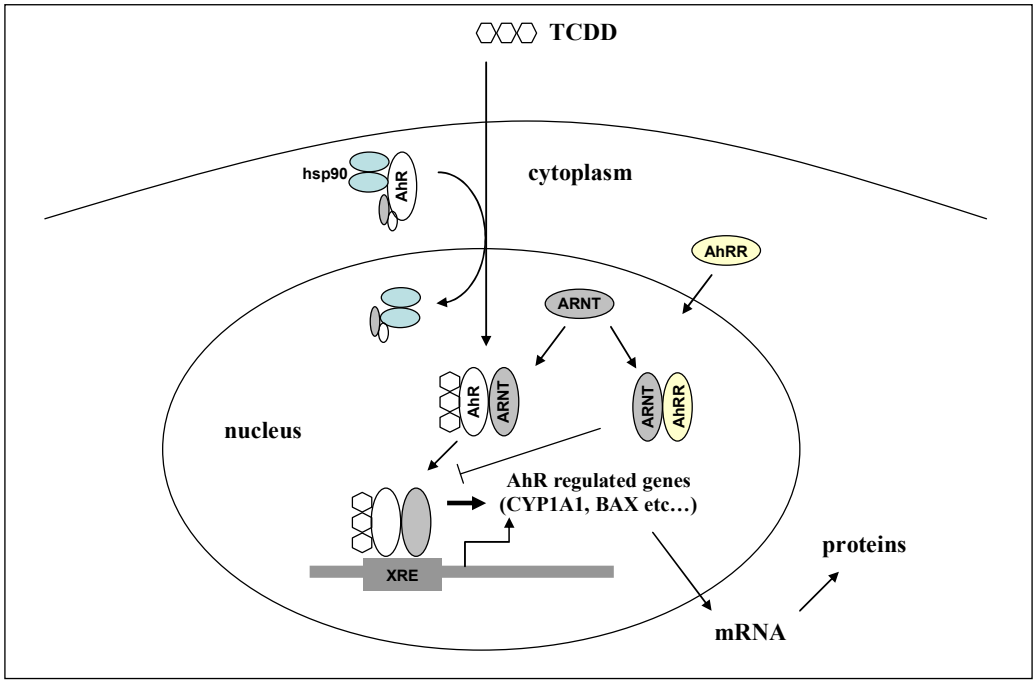

Figure 12. Mechanisms of transcriptional activation by AhR and negative feedback regulation of AhR function by AhRR. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor ; ARNT, aryl hydrocarbon receptor nuclear translocator; AhRR, aryl hydrocarbon receptor repressor; XRE, xenobiotic response element, CYP1A1, cytochrome P450 1A1; BAX, Bcl-2 associated X gene; mRNA, messenger RNA (modified from Mimura and Fujii-Kuriyama 2003, Mimura *et al.*, 1999).

3 AIMS OF THE PRESENT STUDY

Testicular development and maturation are androgen-dependent processes that are known to be very vulnerable to endocrine disruption but the exact roles and mechanisms of action of different chemical compounds are not fully understood. The aim of the present study was to elucidate whether the exposure to endocrine disrupting chemicals during the critical developmental window disrupts the development of fetal rat testis or adrenal gland.

The specific aims of the thesis were:

1. To differentiate the systemic and direct testicular effects of exposure to antiandrogen- and estrogen-like compounds on prenatal rat testicular steroidogenesis by using *in vivo* and *in vitro* exposure models.

2. To study the effects of antiandrogen exposure on Dhh signalling in the developing rat testis.

3. To elucidate the effects of *in utero* exposure to TCDD, DINP and *p,p'*-DDE on fetal male rat steroidogenesis by analyzing plasma hormones and the protein and mRNA expression levels of steroidogenic enzymes and developmental regulatory factors in ED 19.5 fetal male rat testis and adrenal gland.

4 MATERIALS AND METHODS

4.1 ANIMALS

Specific pathogen free (SPF) adult Sprague-Dawley female rats (Hsd:S-D) were housed under controlled conditions with the temperature at 21 ± 3 °C, a relative humidity of 55 ± 15 % and a 12-h lighting cycle. Rats received pelleted standard low soy-content rat chow (RM1/RM3(E) SQC, Special Diet Service, Witham, England in studies I, II and IV; R36, Ewos, Södertelje, Sweden in study III) and water *ad libitum*. The females were paired overnight with males and checked the following morning for the presence of sperm in the vagina. This morning was designated as ED 0.5. Pregnant females were randomly assigned to control groups and treatment groups. The Animal Experiment Committee of the University of Kuopio and The Turku University Committee on the Ethics of Animal Experimentation approved all animal experiments.

4.2 *IN UTERO* EXPOSURE AND THE STUDY DESIGN FOR DEVELOPMENTAL ANALYSIS

Diethylstilbestrol (DES; Sigma Chemical Co., St. Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) and diluted with corn oil (1:25, v/v) was introduced subcutaneously to pregnant rats on ED 13.5, 15.5 and 17.5 at a dose of 20 µg/kg of maternal weight (I).

Flutamide (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in DMSO and diluted in corn oil at 25 mg/ml. The animals were treated once daily by oral gavage with flutamide 25 mg/kg bodyweight from ED 13.5 to 17.5 (I) or from ED 10.5 until sacrifice (ED 14.5, 15.5, 17.5 or 19.5) or delivery (II).

TCDD (UFA-Oil Institute, Ufa, Russia; purity > 99 %) was dissolved in diethyl ether (Merck, Darmstadt, Germany) and adjusted volumes of the solution were mixed with corn oil after which the ether was allowed to evaporate. Dosing solutions were carefully mixed in a magnetic stirrer and sonicated for 20 min before dosing. On ED 11, pregnant rats were treated with a single oral dose of TCDD (0.3 or 1 µg/kg), given in 4 ml/kg corn oil (III).

DINP and ***p,p'*-DDE** were used in study IV: animals were treated once daily by oral gavage with 250 or 750 mg/kg of DINP (Sigma-Aldrich, Steinheim, Germany) or with *p,p'*-DDE (Sigma-Aldrich, Steinheim, Germany) at maternal doses of 50 or 100 mg/kg from ED 13.5 to 17.5. First *p,p'*-DDE was dissolved in DMSO and both *p,p'*-DDE and DINP were further diluted in corn oil.

Control rats in all experiments (I-IV) received vehicle only. Ontogeny of fetal rat testicular steroidogenesis (II) was studied by using male fetuses of non-treated dams.

The dams were sacrificed by cervical dislocation after carbon dioxide anesthesia on ED 14.5, 15.5, 17.5 (II) or 19.5 (I-IV). The body weights of dams were measured after euthanasia. In the TCDD-study (III), maternal liver, kidney and placental weight were also recorded. The uteri were removed and chilled on ice.The number of fetuses, fetal weight, and gender were recorded. Male neonates, at the age of 1 day, were sacrificed by decapitation (II).

4.3 *IN VITRO* ORGAN CULTURES

Testes from 19.5-day-old male rat fetuses (I) were transferred aseptically into four-well culture dishes (Nalge Nunc International, Roskilde, Denmark), one testis per well, containing 1.0 ml Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12 HAM, 15 mM HEPES, 2 mg/l pyridoxine, and 1.2 g/l $NaHCO_3$ (Sigma-Aldrich, Irvine, UK), 0.365 g/l L-glutamine (Life Technologies, Gibco BRL, Paisley, UK), 0.1 % bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), and 0.1 g/l gentamycin. In dose-response experiments, fetal rat testes were incubated for 3 hours in the absence (0 mg/l) or presence of 0.1, 1, 10 and 100 mg/l flutamide or with similar concentrations of DES. The concentration of the solvent (absolute ethanol) in the culture medium was 0.1 % (v/v). Control testes were incubated without chemical treatment. In time-response experiments, testes were incubated for 0.5, 1, or 2 hours in the absence (control) or presence of 100 mg/l flutamide or DES.

In the Dhh signalling study (II), testes with adjacent mesonephroi were explanted from ED 14.5 fetuses in 24-well culture plates and cultured for 3 days on agarose blocks placed in DMEM containing 10% fetal calf serum and 50 µg/ml gentamycin. Testes were cultured in the presence of either 12 nM recombinant mouse sonic hedgehog (rmShh), 25 µM cyclopamine, a steroidal alkaloid that inhibits hedgehog signalling, (Biomol Research Labs, Plymouth Meeting, PA, USA) or vehicle only. In both *in vitro* studies (I-II), testes were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2.

4.4 SAMPLE COLLECTION

Fetal male rat blood samples were collected by axillary puncture into heparinized syringes (II-IV). The blood of male fetuses of the same litter was pooled, kept on ice and centrifuged for 5 min at 1890 x g (III) or 1000 x g (II, IV) at 4 °C. Plasma was stored at -20 °C.

Fetal (I-IV) and neonatal (1-day old) (II) testes were snap-frozen in liquid nitrogen, and stored at -70 °C or at -80 °C Testicular samples were also fixed in Bouin's solution (I), 4 % neutrally-buffered paraformaldehyde (II), and buffered 5 % glutaraldehyde (Merck, Darmstadt, Germany) (IV) for immunohistochemical, histological and ultrastructural studies.

Fetal male rat adrenals (III-IV) at the age of ED 19.5 were snap-frozen in liquid nitrogen, and stored at -70 °C. In study IV, adrenals were also fixed in buffered 5 % glutaraldehyde.

The organ culture medium was collected after 0.5, 1, 2 or 3 hours (I) or after 24, 48 and 72 hours in culture (II) and stored at -20 °C.

4.5 REAL-TIME QUANTITATIVE RT-PCR

Total RNA was extracted from snap-frozen testes (I-IV) and adrenals (III-IV) using the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Two (II) or ten (I, III, IV) micrograms of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMV-RT, 30 units), reverse transcriptase buffer (Promega Corp., Madison, WI, USA), 1 (II) or 2 µg (I, III, IV) oligo(dT)15 Primer (Promega), and 40 units of RNase inhibitor (RNasin, Promega) in a final volume of 25 µl. For quantification of the mRNA levels of selected genes, real-time PCR was performed with specific primer pairs (Table 1; more detailed descriptions in original publications I-IV). The annealing temperature was 57 °C. Real-time PCR reactions were performed using the QuantiTect SYBR Green Rt-PCR Kit (Qiagen) according to the manufacturer's instructions by using the DNA Engine Opticon System (MJ Research, Inc., Waltham, Ma, USA). Expression levels of the respective genes were normalized to the level of housekeeping gene ribosomal protein *S26*.

Table 1. Oligonucleotide primers used in the quantitative PCR measurements.

Gene name	Product size (bp)	GenBank accession no.
StAR	330	NM031558
P450scc	510	NM017286
Hsd3b1	427	M38178
Insl3	313	AF139918
Sf1	230	NM053344
Gata4	327	NM_14473
Dhh	281	XM343327
Ptc1	324	AY357891

4.6 HORMONE MEASUREMENTS

4.6.1 LH

Plasma LH concentrations (II, III, IV) of fetal and neonatal male rats were measured by two-site time-resolved immunofluorometric assay (DELFIA, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) for rat LH (Haavisto *et al*. 1993). The sensitivity of the LH assay was 0.75 pg/tube and the intra-assay coefficient variation (CV) was < 5% at >1 ng/ml and inter-assay CV 7.8 % at 0.78 ng/ml.

4.6.2 Corticosterone

Plasma samples of fetal males were analyzed for corticosterone (III-IV) using a commercial ImmuChem Double Antibody Corticosterone ^{125}I RIA kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA) according to the manufacturer's instructions. The minimum detectable dose of corticosterone was 7.7 ng/ml. Intra-assay CV was 7.1 % and inter-assay CV was 7.2 %.

4.6.3 Progesterone and testosterone

Intratesticular P4 (I) and T (I-IV) levels from diethyl ether-extracted testicular extracts of testicular homogenates and culture media (I-II) were measured by time-resolved fluoroimmunoassay DELFIA (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland). Testes were homogenized by ultraturrax in 0.5 ml ice-cold PBS and 0.1 ml of the homogenate was taken for ether extraction. Ether-extracted samples were reconstituted to 0.1 ml Dilution II buffer (Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Turku Finland) and 25 µl was taken for analysis. The detection level of the assays was 0.1 ng/ml for T and 0.25 ng/ml for P4. The intra-and inter-assay variations were below 6% and 12%, respectively.

4.7 WESTERN BLOT ANALYSIS

Pooled fetal and neonatal testis (I-IV) and fetal adrenals (III-IV) obtained from the same litter were homogenized in ice-cold lysis buffer [0.2 % Nonidet P-40, in phosphate buffered saline (PBS)] containing 1mM phenylmethylsulponyl fluoride (PMSF) (I-II) or a cocktail of protease inhibitors (Complete Mini; Roche Diagnostics, Mannheim, Germany) (III-IV). Homogenates were centrifuged for 15 min at 14 000 rpm at +4 °C. Total protein concentration of the supernatant was measured by the Bradford method (Bio-Rad Laboratories AB, Sundbyberg, Sweden) (I-II) or by the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) (III-IV). Proteins were taken up in sample buffer (100 mM Tris-HCl pH 6.8, DTT 200 mM, PMSF 1 mM, SDS 4 %, glycerol 20 %, bromophenolblue 0.2 %) and equal amounts of protein (20 µg) were separated by 12.5 % SDS-polyacrylamide gel electrophoresis, and electro-blotted onto nitrocellulose or polyvinylidene fluoride membranes (Hybond-P, Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked overnight at +4 °C with PBS containing 3 % non-fat milk powder and 0.3 % Tween 20. Immunochemical detection of the proteins was performed by incubating the membrane for 1 hour at room temperature (RT) in the presence of a specific primary antibody (Table 2) diluted in PBS with 1 % BSA and 0.02 % NaN_3. β-Actin (I-II) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (III-IV) were used as internal loading controls. The same Western blots were used for several antibodies. Antibody binding was detected using corresponding horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Amersham Life Science, Buckinghamshire, UK) for 1 hour at RT, and visualized by ECL chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) on FujiFilm Super RX films. Relative labeling intensities were quantified with Chemi Imager 4400 software (Chemi Imager™, Alpha Innotech Corporation, San Leandro, CA, USA). The Western blot results were presented as relative values compared with the expression level of β-Actin or GAPDH.

Table 2. Antibodies used in Western blot analysis.

Target protein	Primary antibody type and supplier
StAR	polyclonal, donated by Dr. J.F. Strauss III, PA, USA
P450scc	polyclonal, donated by A. H. Payne, Stanford, USA
3β-HSD1	polyclonal, donated by J. I. Mason, Edinburgh, UK
ERα	MC-20), polyclonal, Santa Cruz Biotechnology, CA, USA
AR	(N-20), polyclonal, Santa Cruz Biotechnology, CA, USA
GAPDH	monoclonal, HyTest, Turku, Finland
β-Actin	monoclonal, Signa-Aldrich, St. Louis, MO, USA

4.8 IMMUNOHISTOCHEMISTRY

Bouin's and paraformaldehyde fixed samples from organ cultures were processed for immunohistochemistry (I-II). Fixed samples were embedded in paraffin and cut into 4-µm-thick sections. Deparaffinised sections were pre-incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity and antigen retrieval was performed in 10 mM sodium citrate in a microwave oven. All incubations were carried out at RT. The sections were washed in Tris-buffered saline containing 0.05 % Tween 20 (I) or 0.5% Triton X-100 (II) (TBS/T; pH 7.4). Unspecific antibody binding was blocked by incubating in pre-antibody blocking solution (PowerVision+ Poly-HRP IHC Kit; ImmunoVision Technologies Co., VA, USA) (I) or by preincubating the sections in TBS/T containing 1% BSA and 5% normal goat serum (II) for 30 min. The sections were then incubated with AR antibody (N-20; Santa Cruz Biotechnology, CA, USA) diluted in pre-antibody blocking solution (I) or with cleaved-caspase 3 antibody (Asp175; Cell Signalling Technology, Beverly, MA, USA) diluted in TBS/T containing 0.1% BSA and 0.5% normal goat serum (II) for 1 hour. After washing 3 x 5 min in TBS/T and incubation in post-antibody blocking solution (PowerVision+ Poly-HRP IHC Kit; ImmunoVision Technologies Co.) for 20 min (II) the sections were reacted to poly-horseradish peroxidase anti-Rabbit IgG (PowerVision+ Poly-HRP IHC Kit; ImmunoVision Technologies Co.) (I) and to a goat anti-rabbit secondary antibody diluted in TBS/T containing 0.1% BSA and 0.5% normal goat serum. After 3 washes antibody binding was visualised by reacting the sections to 3,3'-diaminobenzedine according to the manufacturer's instructions (Zymed Laboratories, South San Francisco, CA, USA). Control incubations were carried out by substituting the primary antibody with non-immune rabbit serum.

4.9 LIGHT AND ELECTRON MICROSCOPY

Fetal testicular and adrenal samples, prefixed in glutaraldehyde (IV) were postfixed with potassium ferrocyanide-osmium fixative. The samples were embedded in epoxy resin (Glycidether 100; Merck, Darmstadt, Germany) and cut into sections for light and electron microscopic survey. For viewing by light microscopy, 1-µm-thick sections were stained with 0.5 % toluidine blue. Ultrathin sections (70 nm) were stained with 5 % uranyl acetate (Layrylab, St. Fons, France) and 5 % lead citrate (Laurylab, St.Fons, France) by using Ultrostainer (Leica Corp., Vienna, Austria) and examined with a JEOL 1200 EX electron microscope (Tokyo, Japan).

4.10 STATISTICAL ANALYSIS

In general, the data are presented as litter means ± standard error of mean (SEM). In studies I-III, results from Western blot analysis, real-time quantitative RT-PCR and *in vitro* T and P4 levels are presented as means ± standard error of mean (SEM). Statistical significances between the groups were analysed by analysis of variance (ANOVA) with the post-hoc Dunnett's or Dunn's pairwise multiple comparison *t*-test (I-IV) or by ANOVA with repeated measurements followed by t-tests with Bonferroni correction (II). To test the linear trend in the means of exposure groups (III), ANOVA, followed by Dunnett's or Dunn's pairwise multiple comparison *t* test was performed. A *P*-value <0.05 was adopted as the level of significance.

5 RESULTS

5.1 DEVELOPMENTAL STUDIES

5.1.1 The ontogeny of fetal testicular steroidogenesis and plasma LH (II)

During normal fetal development, intratesticular T concentrations, but not P4, increased with age (II; Figure 2A). On ED 19.5 intratesticular T reached the prenatal peak value ($P < 0.01$) and in neonates, the testicular T content decreased from this level. The fetal testicular P4 content decreased slightly throughout development (II, Figure 2A). Plasma LH levels increased significantly from ED 17.5 to postnatal day 1 (newborns) (II, Figure 2A). The expression of StAR and AR protein paralleled the testicular testosterone profile (II, Figure 2B-C). On ED 19.5, the expression level of StAR protein had elevated 1.7-fold compared with the level on ED 15.5. The expression level of testicular AR followed a similar trend: AR levels increased 1.9-fold on ED 17.5, 3.1-fold on ED 19.5, and 2.2-fold in the newborns compared with the levels on ED 15.5.

5.2 IN UTERO EXPOSURE TO ENDOCRINE DISRUPTERS

5.2.1 Toxicity of chemicals (I-IV)

In utero exposure to DES, flutamide, TCDD, DINP or *p,p'*-DDE did not produce any signs of overt toxicity in the dams as evaluated by body weight gain and visual inspection. TCDD toxicity was also evaluated by weighing the maternal liver, kidney and placenta but the weights of the selected organs were not affected by TCDD-treatment. Litter size and sex ratio were also within normal range in all exposure groups. *In utero* exposure to DES, flutamide, TCDD and *p,p'*-DDE did not affect fetal (I-IV) or neonatal (II) male rat body weight when compared with vehicle-treated control animals. On ED 19.5 the body weight of male fetuses increased significantly in the group treated with 250 mg/kg DINP (IV: Table 3).

5.2.2 Effects on plasma hormone levels (II-III)

Levels of corticosterone, a hormone involved in metabolism and stress responses, tended to increase in 19.5-day-old fetal male rats exposed *in utero* to 0.3 and 1 µg/kg of TCDD and 250 mg/kg of DINP but the increases did not reach statistical significance. Maternal exposure to *p,p'*-DDE had no effect on fetal male corticosterone concentrations. Plasma LH levels, measured from TCDD and *p,p'*-DDE-treated male rat fetuses decreased significantly only at the dose of 1 µg/kg TCDD (III: Figure 5); *in utero* exposure to *p,p'*-DDE did not affect LH levels of fetal male rats on ED 19.5.

5.2.3 mRNA and protein expression levels of steroidogenic enzymes and related factors in fetal testis (I, III, IV)

Exposure to FLU (from ED 13.5 to 17.5) or DES (on EDs 13.5, 15.5 and 17.5) did not alter the protein expression levels of testicular AR (I: Figure 1A and 1C) or StAR (I: Figures 1B-

C) in 19.5-day-old fetal rat. A significant, decreasing linear trend in the protein expression levels of StAR was observed in TCDD-treated male fetuses (III: Figure 3A and 3D) but the expression levels of testicular P450scc and 3β-HSD1 did not decline significantly. ERα and AR protein levels were dose-dependently reduced after TCDD treatment but the changes were not statistically significant (III: Figure 6A-C). *In utero* exposure to DINP did not affect protein levels of testicular StAR, P450scc or 3β-HSD significantly, although 250 mg/kg DINP tended to increase the expression levels of StAR and steroidogenic enzymes (IV: Figure 5). No alterations in testicular AR protein were observed in DINP-treated rats. Maternal exposure to *p,p'*-DDE had no effect on testicular StAR, steroidogenic enzyme or AR protein levels in ED 19.5 testis (IV: Figures. 7 and 8).

To further study the effects of TCDD and DINP on fetal testicular steroidogenesis, transcript levels of *StAR*, *P450scc* and *Hsd3b1* were determined by real-time quantitative RT-PCR. *Hsd3b1* was dose dependently decreased and the difference from the controls became significant at the dose of 1μg/kg TCDD (III: Figure 4). *P450scc* and *StAR* were also dose dependently decreased and the decreasing linear trend following TCDD treatment was significant for *P450scc* (III: Figure 4). A statistically significant increase in testicular transcript levels of *P450scc* was observed at the dose of 750 mg/kg DINP (IV: Figure 3B). No statistically significant changes in relative *StAR* or *Hsd3b1* mRNA levels were seen after DINP as compared to the controls (IV: Figure 3A, 3C). The mRNA expression levels of *Insl3, Gata4* and *Sf1* were not altered by TCDD but DINP-treatment, at a dose of 750 mg/kg, increased significantly the mRNA levels of *Gata4* and *Insl3* (IV: Figure 4B -C).

5.2.3.1 mRNA expression of genes involved in desert hedgehog signalling (II)

Administration of flutamide from ED 10.5 until sacrifice (ED 14.5, 15.5, 17.5 or 19.5) or delivery, significantly decreased the mRNA expression levels of *Dhh* in fetal rat testes on ED 17.5 (II: Figure 3). *Ptc1* expression was significantly suppressed on ED 17.5, 19.5 and in neonates. *Sf1* was also significantly suppressed on ED 17.5 in the flutamide-exposed litters. On ED 14.5 and 15.5, *Sf1* expression was significantly higher than in controls. Expression levels of steroidogenic enzymes *P450scc* and *Hsd3b1* and growth factor *Insl3* were significantly lower in the flutamide-exposed testes than in controls on ED 17.5 and 1 day after birth.

5.2.4 Effects on intratesticular testosterone and progesterone levels (I, III, IV)

P4 and T levels in 19.5-day-old fetal rat testes remained unaltered in the FLU- and DES-treated males (I: Figure 2). Maternal exposure to TCDD decreased testicular T content (III: Figure 2); the decline was significant already with a dose of 0.3 μg/kg TCDD and the value for 1 μg/kg did not differ significantly from this. DINP or *p,p'*-DDE did not alter testicular T content significantly on ED 19.5 (IV: Figure 2). Table 3 summarizes the effects of developmental exposure to endocrine disrupters on testicular steroidogenesis, Insl3 and AR expression and plasma LH levels in male rat fetuses.

Table 3. Summary of the effects of developmental exposures to endocrine disrupters on testicular steroidogenesis, Insl3 and AR expression and plasma LH levels in male rat fetuses.

chemicals	prenatal exposure window (ED)	LH	T	StAR protein	mRNA of steroidogenic enzymes	mRNA of Insl3	AR protein
DES	13.5, 15.5, 17.5	nm	—	—	nm	nm	—
flutamide	13.5-17.5	nm	—	—	nm	nm	—
flutamide	10.5 until sacrifice	nm	nm	nm	⇩	⇩	nm
TCDD	11	⇩	⇩	decreasing tendency	decreasing tendency	—	decreasing tendency
DINP	13.5-17.5	nm	—	—	increasing tendency	⇧	—
p,p-DDE	13.5-17.5	—	—	—	nm	nm	—

ED, embryonic day; LH, luteinizing hormone; StAR, steroidogenic acute regulatory protein; Insl3, insulin-like factor 3; AR, androgen receptor; nm, not measured; —, no effect; ⇩, inhibition; ⇧, stimulation

5.2.5 mRNA and protein expression levels of steroidogenic enzymes and related factors in fetal adrenals (III-IV)

In utero TCDD exposure had no effect on fetal adrenal StAR, P450scc or 3β-HSD1 protein expression levels on ED 19.5 (III: Figure 8A–D). The expression of adrenal ERα and AR protein remained unaltered in TCDD-treated groups (III: Figure 9A-C). The fetal adrenal exposed to 750 mg/kg DINP showed an increasing tendency in the protein expression levels of StAR and P450scc, but it did not reach statistical significance (IV: Figure 6). 3β-HSD1 and AR expression were not significantly altered either (IV: Figure 6). Maternal exposure to *p,p'*-DDE had no effect on fetal adrenal StAR, P450scc, 3β-HSD1 or AR protein levels (IV: Figure 8).

Transcripts of adrenal *Hsd3b1* mRNA were dose dependently decreased, and were 1.7-fold lower at 1 μg/kg TCDD compared with controls (III: Figure 10A). Adrenal *StAR* levels were also dose dependently decreased and those of *P450scc* were decreased at 1μg/kg, but these differences were not statistically significant (III; Figure10A). *Gata4* mRNA levels of TCDD-treated animals did not show significant decreases; however, for *Sf1* mRNA levels, a 1.6-fold decrease in relative transcript levels was observed in the group exposed to TCDD

1 µg/kg compared with the control group (III; Figure 10B). DINP exposure had no effect on adrenal *StAR, P450scc*, *Hsd3b1, Sf1* or *Gata4* transcript levels on ED 19.5.

5.2.6 Effects on testicular and adrenal histology and ultrastructure (IV)

Light microscopic analysis of 19.5-day-old fetal control testis showed normal histology in which fetal-type Leydig cells in the interstitial tissue contained numerous lipid droplets (IV: Figure 9A). At the electron microscopic level, the droplets appeared deeply osmiophilic and the mitochondria had well-developed cristae (IV: Figure 9B). The histology and the ultrastructure of DINP-treated testis did not differ from controls. Contrary to DINP, *in utero* exposure to 100 mg/kg *p,p'*-DDE clearly reduced the number of lipid droplets in Leydig cells and some of them were vacuolated (IV: Figure 9C-D). Adrenals from the control treatment group contained numerous lipid droplets in the cortex as observed by light microscopy (IV: Figure 10A). At the age of ED 19.5, adult-type layers of the cortex (zona fasciculata, reticularis and glomerulosa) were not yet distinct. Ultrastructural analysis of steroidogenic cells of the adrenal cortex showed clusters of lipid droplets and mitochondria, just as steroidogenic active fetal type-Leydig cells in the testis (IV: Figure 10B). Light and electron microscopic observation did not reveal any structural alterations in maternally DINP-exposed fetal adrenals. A maternal dose of 100 mg/kg *p,p'*-DDE reduced the number of lipid droplets remarkably and the cell structure was found to be loose in the cortex (IV: Figure 10C). Ultrastructurally, lipid droplets were small, weakly stained and vacuolated. The smooth endoplasmic reticulum (SER) and part of the mitochondria appeared degenerated in *p,p'*-DDE-treated adrenals (IV: Figure 10D).

5.3 TESTICULAR ORGAN CULTURES AND EXPOSURE TO ENDOCRINE DISRUPTERS

5.3.1 The effects of diethylstilbestrol on testicular steroidogenesis (I)

DES was included in the culture medium for 3 hours at concentrations of 0.1, 1, 10 and 100 mg/l. AR protein expression levels showed a relatively large variation between the individual 19.5-day-old fetal testes exposed to DES 100 mg/l. In some samples the signal was at the limit of detection but due to the large inter-assay variation the change was not statistically significant (I: Figure 7A-B). However, when DES-treated testes were analyzed by AR immunohistochemistry, the labeling intensity was considerably lower throughout the testis compared to control samples, which had strong positive labeling in the nuclei of peritubular myoid cells and in some of the interstitial cells (I: Figure 11). DES at doses of 10 and 100 mg/l seemed to elevate StAR protein expression levels but the change was not statistically significant (I: Figure 7A-B). At the mRNA level, DES had no effect on *StAR* expression compared to controls. Secreted and intratesticular T remained at control level in all exposure groups (I: Figures 8 and 9). However, in the presence of DES 100 mg/l secreted and intratesticular P4 levels increased significantly compared to controls ($P<0.001$) (I: Figures 8 and 9). DES at lower doses (0.1, 1 and 10 mg/l) had no effect on P4 production or accumulation (I: Figures 8 and 9). Time-response experiments, in which testes were cultured

for 0.5, 1 or 2 hours, DES 100 mg/l showed a slight, non-significant decline in secreted T levels (I: Figure 10A). The elevation of secreted P4 was statistically significant ($P<0.01$) in the presence of DES already after 0.5 hours in culture and increased accumulation was also evident ($P<0.001$) after 1 and 2 hours (I: Figure 10B).

5.3.2 The effects of flutamide on testicular steroidogenesis (I)

In the dose-response experiment, a clear decline ($P<0.05$) in AR protein expression of ED 19.5 testis was obtained at a dose of 100 mg/l flutamide (I: Figure 3A-B). A decline in AR expression was also observed by immunohistochemistry: flutamide-exposed testes had very weak labeling intensity compared to controls (I: Figure 11). For StAR protein, a significant increase from the control level occurred at doses of 10 and 100 mg/l corresponding to 3.2- ($P<0.01$) and 4.3-fold ($P<0.001$) elevation, respectively (I: Figure. 3A-B). However, the mRNA expression levels of *StAR* in the flutamide-treated testes did not differ from control level. T secretion in the medium was increased by FLU at 10 and 100 mg/l. The elevation was statistically significant at the dose of 100 mg/l ($P<0.001$) (I: Figure 4). The same was observed for intratesticular T ($P<0.01$) (I: Figure 5). Secreted and intratesticular P4 levels remained similar to the controls (I: Figures 4 and 5). Time-response experiments confirmed the above tendency. The amount of secreted T in medium increased in a time-dependent manner (I: Figure 6A) being 1.4-fold higher in the presence of flutamide than in controls ($P<0.05$) after 2 hours in culture. There was no difference in P4 secretion between the treatment and control groups (I: Figure 6B).

5.3.3 The direct effects of Dhh signalling on testicular development

To test whether impaired Dhh signalling could be responsible for the suppressed gene expression levels observed in the *in vivo* flutamide experiment, we used *in vitro* testis organ cultures where 14.5-day-old fetal rat testis were cultured for 3 days in the presence of vehicle, hedgehog inhibitor cyclopamine, or rmShh, a ligand for Ptc1. Indeed, when fetal testes were cultured in the presence of cyclopamine, they expressed significantly lower levels of not only *Ptc1* but also of *Insl3* and *P450scc* and *Hsd3b1* (II: Figure 4). On the other hand, when fetal testes were cultured in the presence of rmShh, the expression levels of *Ptc1* and *Insl3* but not *P450scc* or *Hsd3b1* were significantly increased compared with testes cultured in the presence of vehicle only. *Sf1* expression showed a similar trend, but this did not reach statistical significance. T secretion by testes cultured in the presence of cyclopamine was significantly lower between 24–48 and 48–72 hours compared with testes cultured in the presence of vehicle (II: Figure 4). Histological and immunohistochemical analysis of the testis-mesonephros organ cultures showed that after 3 days in culture, the Wolffian duct was well developed in the vehicle and rmShh-exposed samples, whereas in the cyclopamine-exposed cultures, it had remained closed (II: Figure 5A-C). In the latter, the efferent ducts appeared widened and the epithelium was flattened. Moreover, the cyclopamine-exposed testes partly maintained the Müllerian duct, which had regressed in the vehicle and rmShh-exposed testes. 3β-HSD1 immunohistochemistry suggested a reduced number of steroidogenic Leydig cells after cyclopamine treatment. To determine whether

this decrease was due to increased apoptosis, the cultures were subjected to cleaved-caspase 3 immunohistochemistry. Although no clear conclusion regarding the number of apoptotic Leydig cells could be drawn, cleaved-caspase 3 immunostaining revealed extensive apoptosis in the Wolffian ducts when cultured in the presence of cyclopamine but not in the controls or rmShh-treated organ cultures (II: Figure 5D-F). Interestingly, a similar expression pattern of cleaved-caspase 3 as observed in the cyclopamine-treated testes was observed in non-treated fetal ovary cultures.

6 DISCUSSION

6.1 TESTICULAR STEROIDOGENESIS DURING PRENATAL LIFE

In mammals, the development of the male phenotype is critically dependent on androgens (Wilson 1978). During normal fetal development, intratesticular T levels increased steadily from ED 15.5 onward with a peak on ED 19.5 and a subsequent decline 1 day after birth. This was paralleled by increased AR and StAR expression. Using immunohistochemistry, we have observed weak AR immune reactivity in peritubular myoid cells and interstitial cells earliest at the on ED 15.5 (data not shown). AR immune reactivity has been detected in fetal rats as early as on ED 14.5 in interstitial cells and peritubular myoid cells, but it is absent from fetal Sertoli cells (You & Sar 1998). Using double immunohistochemistry, Mylchreest and co-workers (Mylchreest *et al*. 2002) have convincingly identified the presence of AR and 3β-HSD1 double-positive fetal Leydig cells in the rat testis on ED 16 and 18. However, others did not observe this co-localization and argued that AR is not expressed in fetal Leydig cells but localizes to the interstitial cells surrounding the seminiferous cords from ED 17 onward (Majdic *et al*. 1995). Concomitantly, LH levels steadily elevated from ED 17.5 up to 1 day after birth, reflecting the proliferation and functional differentiation of fetal Leydig cells that are responsible for the production of androgens and consequent development of the male reproductive tract. In male rats, the negative feedback regulation of gonadotropins starts functioning from ED 19.5 onwards (Pakarinen *et al*. 1994).

6.2 CRITICAL DEVELOPMENTAL WINDOW FOR EXPOSURE TO ENDOCRINE DISRUPTERS

Various xenobiotics and naturally occurring compounds have been found to disrupt the endocrine system of animals. Disorders in sex steroid balance during fetal development may interfere with male reproductive success and health later in life (Sharpe 2001). Reduction in androgen-dominance to estrogens and interference with androgen action are apparent mechanisms causing demasculinization (Sharpe 2001, Rivas *et al*. 2002b).

It has been shown that androgen driven masculinization is programmed by androgen action during a common early programming window that is prior to ED 19.5 in rat (Welsh *et al*. 2008). When pregnant Wistar rats were treated with AR antagonist, flutamide, 100 mg/kg of maternal body weight once a day during the early programming window, a period just after onset of testicular T production (from ED 15.5 to 17.5), or during the middle window that is the period immediately prior to morphological differentiation of reproductive tract tissues (from ED 17.5 to 19.5), both exposure setups reduced AGD and phallus length near to female levels in postnatal males (Welsh *et al*. 2008). Flutamide exposure also prevented prostate and seminal vesicle formation in almost all males at PND 25. Exposure to flutamide during the late programming window, from ED 19.5 to 21.5, had no effect on the formation of the male reproductive tract tissues, as all reproductive tissues were present in all males (Welsh *et al*. 2008). In the present study, all *in utero* exposures were carried out during

the critical developmental window, except for the rats exposed to TCDD. Because of the lipophilic properties and slow elimination of the compound, the dosing treatment on ED 11 was sufficient to cover the critical period for TCDD-induced reproductive toxicity in the male rat fetuses (Li *et al*. 1995).

In utero exposure to DES (on EDs 13.5, 15.5 and 17.5) and flutamide (from ED 13.5-17.5) had no obvious effects on ED 19.5 rat testicular T and P4 production, StAR protein or AR protein expression (I). However, exposure to flutamide from ED 10.5 until sacrifice on ED 17.5 or delivery, significantly decreased the expression levels of steroidogenic enzymes *P450scc* and *Hsd3b1* and growth factor *Insl3* in fetal and neonatal rat testis indicating disrupted fetal Leydig cell function (II). In addition to decreased steroidogenic enzyme levels, the expression of *Dhh*, *Ptch1* and *Sf1* were down-regulated on ED 17.5. In the fetal mouse testis, Sf1 expression marks the proliferative phase of pre-Sertoli cells and fetal Leydig cells (Schmahl *et al*. 2000). The observed changes in the flutamide-exposed fetal rat testicular gene profile suggests that somatic cell differentiation could also be impaired. In a number of studies, androgen dependent reproductive disorders and decreased androgen levels have been reported in perinatally DES or flutamide-exposed rodents (McIntyre *et al*. 2001, McKinnell *et al*. 2001, Guyot *et al*. 2004). The doses used in the study, DES 20 µg/kg and flutamide 25 mg/kg of maternal body weight, were also selected based on their known ability to disrupt the normal androgen action and thereby testis development (McIntyre *et al*. 2001, Guyot *et al*. 2004). However, *in utero* exposure to DES and flutamide does not necessarily show overt effects in fetal testis despite the fact that they induce permanent changes in developing testis and accessory sex organs that may appear as late effects.

The sensitivity of the male rat reproductive system to TCDD is greatly influenced by the timing of exposure (Bjerke & Peterson 1994, Ohsako *et al*. 2002). Our results suggest that TCDD treatment as early as on ED 11 interferes with the regulation of testicular steroidogenesis and decreases steroidogenesis in 19.5-day-old fetal rat testis: a maternal dose of 0.3 µg/kg TCDD caused a significant decrease in the testicular T content of 19.5-day-old fetal rat (IIII). Exposure to TCDD also tended to reduce the mRNA and protein expression levels of testicular StAR and steroidogenic enzymes P450scc and 3β-HSD1. The number of animals used in this experiment was rather low and it is possible that a significant reduction in steroidogenic enzyme levels could have been obtained by exposing more animals in each treatment group. One possible explanation for the TCDD-induced decrease in testicular steroidogenesis could be the reduction in Leydig cell number. In adult male rats exposed to TCDD, the size and the number of Leydig has been decreased (Johnson *et al*. 1994). ERα and AR protein expression in ED 19.5 male rat testis tended to be reduced by maternal TCDD exposure. Similar results have also been demonstrated earlier in fetal male rats (Mutoh *et al*. 2006). It was recently shown that dioxins exert their effect by forming atypical enzyme complexes that mediate the breakdown of steroid hormone receptors (Ohtake *et al*. 2007). This could explain the reduction in steroid receptor levels, but further research is required to fully delineate the molecular interactions that occur between the ER, AR, and AhR as well as their dose–response relationships. The mechanisms underlying the TCDD-induced impaired steroidogenesis are complex and not fully understood. Despite the view that

fetal pituitary gonadotropins are the initial target of dioxin (Mutoh *et al*. 2006) our results indicate that LH production is affected by TCDD only at rather high doses; fetal male rat LH levels were significantly reduced at 1 µg/kg TCDD. Only minor alterations in steroidogenesis were observed in ED 19.5 adrenal after TCDD exposure.

Developmental exposure to environmental antiandrogen DINP from ED 13.5 to 17.5 did not down-regulate the activity of testicular or adrenal steroidogenesis in 19.5-day old fetal male rat (IV). Neither histological nor ultrastructural studies revealed changes in the testes or adrenals of DINP-treated rats compared with controls. It has been shown previously that prenatal exposure to phthalate esters inhibits T synthesis and *Insl3* expression in fetal Leydig cells (Parks *et al*. 2000, Barlow *et al*. 2003, Wilson *et al*. 2004) and phthalate esters do not act through ARs (Mylchreest *et al*. 1999, Parks *et al*. 2000)**.** However, DINP seems to have a lower antiandrogenic potency than some other phthalates, such as DEHP, DBP or benzyl butyl phthalate (BBP) (Gray *et al*. 2000, Barlow *et al*. 2004, Borch *et al*. 2004). Nevertheless, *in utero* exposure to a high dose of DINP (750 mg/kg) from ED 7 to ED 21 has been reported to decrease testicular T production *ex vivo* and T content of the 21-day-old fetal male rat (Borch *et al*. 2004). In the present study, 750 mg/kg of DINP from EDs 13.5 to 17.5 caused elevated testicular mRNA levels of *P450scc*, *Gata4* and *Insl3* on ED 19.5 although no effects on intratesticular T levels or protein levels of testicular StAR, P450scc or 3β-HSD1 were detected. These observed increases in the expression levels of *P450scc*, *Gata4 and Insl3* could be due to a rebound effect on steroidogenesis. The reason for the different outcome in our study and in that of Borch and co-workers (Borch *et al*. 2004) may be the shorter exposure time that we used; steroidogenesis may have recovered during the two day interval between the last exposure and analyses. This view is supported by the study in which 48 h after the cessation of DBP exposure fetal rat testicular mRNA levels of *StAR* and steroidogenic enzymes returned to normal as toxic phthalate was cleared from the system (Thompson *et al*. 2004).

In contrast to DINP, *p,p'*-DDE is known to act as a classic antiandrogenic compound similar to flutamide; it blocks androgen binding to the AR (Freyberger *et al*. 2007). It has been suggested that *p,p'*-DDE could also affect androgen-sensitive tissues by stimulating conversion of T to less active metabolites. However, here we report that *p,p'*-DDE *in utero* did not alter testicular T concentrations or the protein expression levels of AR, StAR, P450scc or 3β-HSD1 (IV). In the present study, no significant changes were observed in male rat plasma LH or corticosterone levels. In the adrenal, no alterations in the protein expression levels of StAR or steroidogenic enzymes were detected. Despite the fact that there were no differences in steroidogenic outcomes in *p,p'*-DDE-treated rat fetuses compared with the controls, histological and ultrastructural analyses revealed severe alterations in the structure of steroidogenic cells. Metabolic activation of 3-methylsulphonyl-DDE ($MeSO_2$-DDE), another DDT metabolite, causes severe vacuolation and degeneration of the mitochondria in adrenocortical cells of fetal mouse treated *in utero* (Jonsson *et al*. 1995). However, it has been shown that there are striking species differences in the binding of $MeSO_4$-DDE to adrenocortical tissue; a high binding in mouse adrenal cortex was confirmed while binding in rat adrenal cortex was very weak (Lindstrom *et al*. 2008). It has been suggested that the

mitochondrial degeneration in the adrenocortical cells, an effect also observed in our study with *p,p'*-DDE, is the result of mitochondrial P450c11-catalyzed metabolic activation in the adrenal cortex (Jonsson *et al*. 1991, Lund & Lund 1995). Due to enzyme specificity, *p,p'*-DDE accumulates into mitochondria wherein it blocks glucocorticoid synthesis. It is also known to inhibit oxidative phosphorylation (Ferreira *et al*. 1997) and thus increase the permeability of mitochondrial membranes. This in turn may lead to ruptured mitochondrial structure. It can be foreseen that high levels of maternal corticosterone could have maintained the corticosterone levels of *p,p'*-DDE-treated fetuses that were unaltered despite all histological and ultrastructural changes in adrenocortical cells. However, no changes in steroidogenic enzymes were observed despite apparent ultrastructural changes in exposed animals. It has been shown that unlike the adult rat, fetal rat testis expresses the mRNA of P450c11 (Hatano *et al*. 1996). Thus, in addition to the cytotoxic effects of *p,p'*-DDE on fetal adrenocortical cells it can be presumed that fetal testis could also be a target of *p,p'*-DDE activity.

Several studies have pointed to the importance of androgens on fetal Sertoli and Leydig cell function (Abel *et al*. 2008, Scott *et al*. 2008, Welsh *et al*. 2008). For example, both the androgen-insensitive *Tfm* mouse, which carries an inactivating mutation in the AR, as well as the AR-knockout mouse exhibit reduced numbers of Sertoli cells just after birth (Johnston *et al*. 2004, Tan *et al*. 2005). Thus, androgenic effects on fetal Leydig cells and fetal Sertoli cells are mediated either directly through AR-positive fetal Leydig cells or, alternatively, through the interstitial peritubular myoid cells that express AR during fetal development. Differentiation of these peritubular myoid cells is induced by Sertoli cell-derived Dhh (Yao *et al*. 2002). If the peritubular myoid cells are the primary targets of androgen action, then they must signal to the Sertoli cells in a paracrine manner where they regulate Dhh expression. In turn, Dhh secretion would induce differentiation of not only the peritubular myoid cells but also of the fetal Leydig cells. In either case, exposure to antiandrogens has potential to interfere with these signalling pathways, resulting in impaired fetal Leydig cell differentiation and suppressed levels of steroidogenic enzymes and *Insl3*. Presumably, this leads to insufficient levels of T and INSL3, which prevents full masculinization. However, in our studies, testicular AR levels were not altered in male rats treated with classic AR antagonist flutamide or *p,p'*-DDE. It can be assumed that exposure to these chemicals might not have caused a total block of AR, which might also explain why we did not see any dramatic effects.

6.3 COMPARISON OF *IN VIVO* AND *IN VITRO* EXPOSURE

The endocrine system exhibits an organizational effect on the developing embryo. To understand the direct developmental consequences of endocrine disrupters in the testis, we determined the steroidogenic effects of flutamide and DES on ED 19.5 rat testis *in vitro* (I). Flutamide induced a rapid down-regulation of AR protein levels and increased steroidogenic activity, seen both at the level of T production, and StAR protein expression, in testis cultured for 3 hours. All this may result from the autocrine role of ARs in fetal Leydig cell function. Steroidogenic activity in Leydig cells is directly correlated to the presence of

3.4, 2.7 and 1.6 kb *StAR* mRNA transcripts (Stocco & Clark 1996). However, in the present study, no differences in relative expression levels of *StAR* transcripts were observed. Stocco and Clark (Stocco & Clark 1996) have suggested that new RNA synthesis may not be required for the acute or short-term stimulation of steroidogenesis. However, transcription may be essential for the long-term maintenance of steroid production. In a time-response study an increasing trend in T levels was observed in the presence of flutamide.

In DES-treated testis cultures P4 accumulated in media most probably since T synthesis was partially inhibited. DES interferes with steroid production in the testis by inhibiting the expression and activity of P450c17 enzyme (Majdic *et al*. 1996) (I). Our finding that a high dose of DES (100 mg/l) decreased the AR protein expression level is similar to studies conducted *in vivo* in neonatal rats (McKinnell *et al*. 2001, Rivas *et al*. 2002b). Decrease in AR protein level could be due to the toxic effects of high doses of DES and disruption in the androgen-estrogen balance. In a study by Haavisto and others (Haavisto *et al*. 2003), fetal testes cultured for 3 hours in the presence of DES 100 mg/l, revealed alterations in the ultrastructure of Leydig cells; mitochondria and tubules of smooth endoplasmic reticulum were enlarged and lipid droplets were surrounded by accumulation of membrane vesicles. The significance of these findings remained unclear. *In vitro* studies with DES indicate a rapid potential of fetal testis to respond to endocrine disrupters.

6.4 DESERT HEDGEHOG SIGNALLING IN DEVELOPING RAT TESTIS

The signalling pathways that induce differentiation of fetal Leydig cells are largely unknown, but studies in mice have provided evidence that Dhh/Ptc1 signalling triggers fetal Leydig cell differentiation through up-regulation of *Sf1* and *P450scc* (Yao *et al*. 2002). Dhh is expressed and secreted by the somatic Sertoli cells (Bitgood *et al*. 1996) and binds to Ptc1 receptors that are expressed by peritubular myoid cells and possibly interstitial Leydig cells (Clark *et al*. 2000). Sf1 is a nuclear transcription factor that regulates the transcription of a wide variety of genes that are involved in the development of steroidogenic tissues (Val *et al*. 2003), including genes that encode StAR and the steroidogenic enzymes P450scc, 3β-HSD1, and P450c17 (Morohashi *et al*. 1992, Leers-Sucheta *et al*. 1997, Reinhart *et al*. 1999). Moreover, in a recent study, Park and others (Park *et al*. 2007) showed that Dhh-driven Leydig cell development in the mouse is directly dependent on Sf1 action. It should be noted that thus far, to our knowledge, data regarding the role and function of Dhh signalling in fetal rat testis development are lacking.

When Dhh signalling was inhibited in fetal testis-mesonephros organ cultures, it had the same effects on fetal Leydig cell maturation as exposure to flutamide (II). In organ cultures, cyclopamine, which is a specific inhibitor of hedgehog signalling, caused decreased levels not only of *Ptc1* but also of growth factor *Insl3* and steroidogenic enzymes *P450scc* and *Hsd3b1*. Shh acts in a similar manner as Dhh and therefore rmShh was used as a stimulator *in vitro* studies of hedgehog signalling. When Dhh signalling was stimulated by rmShh, *Insl3* expression was increased. Although no effect was seen on the mRNA expression of *P450scc* and *Hsd3b1*, T secretion by the cyclopamine-exposed testis cultures was severely affected.

Organ cultures that were exposed to vehicle typically showed a well-developed Wolffian duct. The cultures treated with cyclopamine, on the other hand, were characterized by a closed duct that showed strong expression of cleaved-caspase 3, a marker of apoptosis, reflecting that T is necessary for the maintenance of the Wolffian duct. In a recent study, Welsh and others (Welsh *et al*. 2008) showed that Wolffian duct development is a biphasic process and that flutamide exposure *in utero* specifically during the early programming window from ED 15.5–17.5 resulted in incomplete or absent Wolffian ducts in adult male rats. These findings fit well with our results obtained in the *in vitro* organ cultures where ED 14.5 testes were cultured for 3 subsequent days. Moreover, in testis cultures that had been exposed to cyclopamine, remnants of the Müllerian duct could still be discerned. In the vehicle- or rmShh-exposed testis, these remnants were absent, suggesting that inhibition of hedgehog signalling additionally impairs the production of anti-Müllerian hormone by the Sertoli cells. The mechanism underlying this effect is not known; future studies are required.

7 CONCLUSIONS

The present study was performed to investigate the effects of endocrine disrupters on fetal male rat testicular and adrenal development *in vivo* and also to differentiate the systemic and direct testicular effects of endocrine disrupters by performing *in vitro* cultures. The main conclusions of the present work are the following:

1. DES and flutamide are able to induce rapid steroidogenic changes in fetal rat testis under *in vitro* conditions. Although *in utero* exposure to these chemicals does not necessarily show overt effects in fetal testis, they can induce permanent changes in developing testis and accessory sex organs later in life.

2. Exposure to antiandrogens can interfere with Dhh signalling and result in the impaired differentiation of the fetal Leydig cells and subsequently lead to abnormal testicular development and sexual differentiation.

3. *In utero* exposure to TCDD has direct testicular and pituitary effects on fetal male rat but with different dose-responses. These changes can lead to impaired steroidogenesis and may result in maldevelopment of the testis and thereby weaken masculinization.

4. *In utero* exposure to environmental antiandrogens, DINP or *p,p'*-DDE did not down-regulate testicular or adrenal steroidogenesis in 19.5 day-old fetal rat. However, *p,p'*-DDE-treatment caused clear histological and ultrastructural changes in the prenatal testis and adrenal gland. These structural alterations can disturb the development and function of fetal testis and adrenal gland that may become evident later in life.

8 ACKNOWLEDGEMENTS

This work was mainly carried out at the Department of Physiology, Institute of Biomedicine, University of Turku during the years 2005-2009. In addition, parts of the work were done at the Laboratory of Animal Physiology, University of Turku, Laboratory of Electron Microscopy, University of Turku and at the Department of Environmental Health, Laboratory of Toxicology, National Institute for Health and Welfare, Kuopio. This thesis would not have been finished but for the good collaboration with these laboratories!

I owe my deepest gratitude to my supervisors Professor Jorma Toppari and Docent Jorma Paranko who introduced me to the topic of endocrine disruption and testis development. I wish to thank Jorma Toppari for the opportunity to complete my thesis work in his innovative and warm-spirited group. I truly appreciate your never-failing enthusiasm to research. Thank you for your careful guidance. I am also grateful to Jorma Paranko for his scientific knowledge and support during the progress of this thesis.

I want to thank the reviewers, Professor Raimo Pohjanvirta and Docent Krista Erkkilä for their expert review of this thesis. Michael Nelson, PhD, is acknowledged for the careful work with revision of the language.

Professor Lauri Pelliniemi is acknowledged for stimulating scientific discussions. I also wish to thank the entire personnel at the Laboratory of Electron Microscopy for good and inspiring coffee break discussions and for all technical help.

My special thanks go to Kuopio: Docent Matti Viluksela and Ulla Simanainen, PhD, are warmly acknowledged for their great contribution to the TCDD work. I also want to thank all other members of the Laboratory of Toxicology and skilful technical personnel in Kuopio. I have always felt welcome when I have visited the laboratory.

I express my sincere gratitude to Professor Mikko Nikinmaa and the personnel at the Laboratory of Animal Physiology. It has been a joy to visit the campus at the University Hill and do experiments in your lab. Thanks for all help!

I wish to thank Virpi Salonen for her great help with *p,p'*-DDE experiment, nice lunch breaks and good discussions. Leon Brokken, PhD, is acknowledged for friendship and perfect teamwork! Here, I also want to mention Groove FM: ”The best radio station in Finland”.

My most sincere thanks go to my friend and colleague Mirja Nurmio. We have had a good time together. You have been sharing “this all”: you have been my support person, help desk and therapist during these years. I think you could add that all on your CV! I am so happy that we are sharing the party together. Thank you for everything.

I owe my deep thanks to Professor Matti Poutanen for good scientific discussions and for providing valuable advice during these years. I wish to thank all the current and former colleagues and technical personnel at the Department of Physiology for making the working

atmosphere so nice. I wish to thank Taija Saloniemi, Sonia Bourguiba, PhD, Helena Virtanen, MD, PhD, Heli Koivuniemi, Heikki Turunen, Jenni Paviala, Juho Mäkelä, Anu Sironen, PhD, Docent Noora Kotaja, Adolfo Rivero-Müller, PhD, and Oliver Meikar for the companionship at the Department of Physiology. I am also grateful to Anneli Vesa, Leena Karlsson, Marjatta Keistinen, Marko Tirri, Tuula Hämäläinen, PhD, Johanna Järvi, Pirjo Pakarinen, PhD, Eila Virransuo, Hannele Rekola, Jonna Palmu, Taija Leinonen, Erica Nyman and to Taina Kirjonen who have always kindly helped me whatever the problem has been.

I owe my warm thanks to Tapio Haavisto, PhD, who has introduced me into the field of endocrine disruption in practice. I truly appreciate your patience. You are a great teacher!

My special thanks go to Sari Pesonen, PhD, Elina Haavisto and Tiina Mikkilä, my friends and former colleagues working with endocrine disruption. You are my peer support!

I want to thank all my dear friends for proving support and great companionship! I also want to mention the badly behaving dogs Elli and Tapio for keeping me moving.

I want to give my loving thanks to my parents Kaija and Raimo for all their support and encouragement. I also want to thank Maj-Britt for her care. My sister Erika and her family are acknowledged for their support and taking my thoughts away from the scientific world. It is absolutely impossible to have some rational thoughts when Lennart wants to “saw or repair” something meanwhile the twins, Alvar and Astrid, are crawling in different directions and at least one is putting foreign bodies into their mouth.

Finally, I want to thank you, Tommi. Lately, you have taken care of most of the housework (you have even fed my aquarium fish). But I promise to become a better wife after the dissertation! Thanks for your love and support. You are my everything.

This work was financially supported by the Academy of Finland, the Turku University Foundation, the Sigrid Jusélius Foundation, the Yrjö Jahnsson Foundation, the Turku University Hospital, the Finnish Cultural Foundation, the Finnish Concordia Fund and the European Commission.

Turku, May, 2009

REFERENCES

Abel, M. H., Baker, P. J., Charlton, H. M., Monteiro, A., Verhoeven, G., De Gendt, K., Guillou, F., and O'Shaughnessy, P. J. (2008). Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology.* 149, 3279-3285.

Adami, H. O., Bergstrom, R., Mohner, M., Zatonski, W., Storm, H., Ekbom, A., Tretli, S., Teppo, L., Ziegler, H., and Rahu, M. (1994). Testicular cancer in nine northern European countries. *Int. J. Cancer.* 59, 33-38.

Anderson, D. J. (1993). Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. *Annu. Rev. Neurosci.* 16, 129-158.

Andersson, S. (1995). 17 Beta-Hydroxysteroid Dehydrogenase: Isozymes and Mutations. *J. Endocrinol.* 146, 197-200.

Aubert, M. L., Begeot, M., Winiger, B. P., Morel, G., Sizonenko, P. C., and Dubois, P. M. (1985). Ontogeny of hypothalamic luteinizing hormone-releasing hormone (GnRH) and pituitary GnRH receptors in fetal and neonatal rats. *Endocrinology.* 116, 1565-1576.

Axmon, A. and Rignell-Hydbom, A. (2006). Estimations of past male and female serum concentrations of biomarkers of persistent organochlorine pollutants and their impact on fecundability estimates. *Environ. Res.* 101, 387-394.

Baba, T., Mimura, J., Nakamura, N., Harada, N., Yamamoto, M., Morohashi, K., and Fujii-Kuriyama, Y. (2005). Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. *Mol. Cell. Biol.* 25, 10040-10051.

Babich, M. A., Chen, S. B., Greene, M. A., Kiss, C. T., Porter, W. K., Smith, T. P., Wind, M. L., and Zamula, W. W. (2004). Risk assessment of oral exposure to diisononyl phthalate from children's products. *Regul. Toxicol. Pharmacol.* 40, 151-167.

Badawi, A. F., Cavalieri, E. L., and Rogan, E. G. (2000). Effect of chlorinated hydrocarbons on expression of cytochrome P450 1A1, 1A2 and 1B1 and 2- and 4-hydroxylation of 17beta-estradiol in female Sprague-Dawley rats. *Carcinogenesis.* 21, 1593-1599.

Baram, T. Z. and Lerner, S. P. (1991). Ontogeny of corticotropin releasing hormone gene expression in rat hypothalamus--comparison with somatostatin. *Int. J. Dev. Neurosci.* 9, 473-478.

Barlow, N. J., McIntyre, B. S., and Foster, P. M. (2004). Male reproductive tract lesions at 6, 12, and 18 months of age following *in utero* exposure to di(n-butyl) phthalate. *Toxicol. Pathol.* 32, 79-90.

Barlow, N. J., Phillips, S. L., Wallace, D. G., Sar, M., Gaido, K. W., and Foster, P. M. (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. *Toxicol. Sci.* 73, 431-441.

Beard, J., Sladden, T., Morgan, G., Berry, G., Brooks, L., and McMichael, A. (2003). Health impacts of pesticide exposure in a cohort of outdoor workers. *Environ. Health Perspect.* 111, 724-730.

Bielinska, M., Kiiveri, S., Parviainen, H., Mannisto, S., Heikinheimo, M., and Wilson, D. B. (2006). Gonadectomy-induced adrenocortical neoplasia in the domestic ferret (Mustela putorius furo) and laboratory mouse. *Vet. Pathol.* 43, 97-117.

Bielinska, M., Seehra, A., Toppari, J., Heikinheimo, M., and Wilson, D. B. (2007). GATA-4 is required for sex steroidogenic cell development in the fetal mouse. *Dev. Dyn.* 236, 203-213.

Birnbaum, L. S. (1994). The mechanism of dioxin toxicity: relationship to risk assessment. *Environ. Health Perspect.* 102 Suppl 9, 157-167.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* 172, 126-138.

Bitgood, M. J., Shen, L., and McMahon, A. P. (1996). Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr. Biol.* 6, 298-304.

Bjerke, D. L. and Peterson, R. E. (1994). Reproductive toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in male rats: different effects of *in utero* versus lactational exposure. *Toxicol. Appl. Pharmacol.* 127, 241-249.

Block, K., Kardana, A., Igarashi, P., and Taylor, H. S. (2000). *In utero* diethylstilbestrol (DES) exposure alters Hox gene expression in the developing mullerian system. *FASEB J.* 14, 1101-1108.

Bock, K. W. and Kohle, C. (2006). Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem. Pharmacol.* 72, 393-404.

Boisen, K. A., Main, K. M., Rajpert-De Meyts, E., and Skakkebaek, N. E. (2001). Are male reproductive disorders a common entity? The testicular dysgenesis syndrome. *Ann. N. Y. Acad. Sci.* 948, 90-99.

Borch, J., Ladefoged, O., Hass, U., and Vinggaard, A. M. (2004). Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. *Reprod. Toxicol.* 18, 53-61.

Bose, H. S., Sugawara, T., Strauss, J. F.,3rd, and Miller, W. L. (1996). The pathophysiology and genetics of congenital lipoid adrenal hyperplasia. International Congenital Lipoid Adrenal Hyperplasia Consortium. *N. Engl. J. Med.* 335, 1870-1878.

Boudouresque, F., Guillaume, V., Grino, M., Strbak, V., Chautard, T., Conte-Devolx, B., and Oliver, C. (1988). Maturation of the pituitary-adrenal function in rat fetuses. *Neuroendocrinology.* 48, 417-422.

Brunnberg, S., Pettersson, K., Rydin, E., Matthews, J., Hanberg, A., and Pongratz, I. (2003). The basic helix-loop-helix-PAS protein ARNT functions as a

potent coactivator of estrogen receptor-dependent transcription. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6517-6522.

Byskov, A. G. (1986). Differentiation of mammalian embryonic gonad. *Physiol. Rev.* 66, 71-117.

Calafat, A. M., Brock, J. W., Silva, M. J., Gray, L. E.,Jr, Reidy, J. A., Barr, D. B., and Needham, L. L. (2006). Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-n-butyl phthalate. *Toxicology.* 217, 22-30.

Capel, B., Albrecht, K. H., Washburn, L. L., and Eicher, E. M. (1999). Migration of mesonephric cells into the mammalian gonad depends on Sry. *Mech. Dev.* 84, 127-131.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ.* 305, 609-613.

Caron, K. M., Soo, S. C., Wetsel, W. C., Stocco, D. M., Clark, B. J., and Parker, K. L. (1997). Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11540-11545.

Carreau, S., Bourguiba, S., Lambard, S., Galeraud-Denis, I., Genissel, C., and Levallet, J. (2002). Reproductive system: aromatase and estrogens. *Mol. Cell. Endocrinol.* 193, 137-143.

Cederroth, C. R., Schaad, O., Descombes, P., Chambon, P., Vassalli, J. D., and Nef, S. (2007). Estrogen receptor alpha is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism. *Endocrinology.* 148, 5507-5519.

Christiansen, S., Scholze, M., Axelstad, M., Boberg, J., Kortenkamp, A., and Hass, U. (2008). Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat. *Int. J. Androl.* 31, 241-248.

Clark, A. M., Garland, K. K., and Russell, L. D. (2000). Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biol. Reprod.* 63, 1825-1838.

Cocco, P., Kazerouni, N., and Zahm, S. H. (2000). Cancer mortality and environmental exposure to DDE in the United States. *Environ. Health Perspect.* 108, 1-4.

Couse, J. F., Dixon, D., Yates, M., Moore, A. B., Ma, L., Maas, R., and Korach, K. S. (2001). Estrogen receptor-alpha knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. *Dev. Biol.* 238, 224-238.

Couse, J. F. and Korach, K. S. (2004). Estrogen receptor-alpha mediates the detrimental effects of neonatal diethylstilbestrol (DES) exposure in the murine reproductive tract. *Toxicology.* 205, 55-63.

Delaere, K. P. and Van Thillo, E. L. (1991). Flutamide monotherapy as primary treatment in advanced prostatic carcinoma. *Semin. Oncol.* 18, 13-18.

Dieckmann, M. J., Davis, M. E., Rynkiewicz, L: M. L:, and Pottinger, R. E. (1953). Does the administration of diethylstilbestrol during pregnancy have therapeutic value? *Am. J. Obstet. Gynecol.* 66, 1062-1081.

El-Gehani, F., Zhang, F. P., Pakarinen, P., Rannikko, A., and Huhtaniemi, I. (1998). Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biol. Reprod.* 58, 116-123.

Elliott, J. E., Norstrom, R. J., and Keith, J. A. (1988). Organochlorines and eggshell thinning in northern gannets (*Sula bassanus*) from Eastern Canada, 1968-1984. *Environ. Pollut.* 52, 81-102.

Else, T. and Hammer, G. D. (2005). Genetic analysis of adrenal absence: agenesis and aplasia. *Trends Endocrinol. Metab.* 16, 458-468.

Ema, M. and Miyawaki, E. (2001). Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. *Reprod. Toxicol.* 15, 189-194.

Emmen, J. M., McLuskey, A., Adham, I. M., Engel, W., Verhoef-Post, M., Themmen, A. P., Grootegoed, J. A., and Brinkmann, A. O. (2000). Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism. *Endocrinology.* 141, 846-849.

European Union Risk Assessment Report. http://ecb. jrc. it/ Documents/Existing-Chemicals/RISK_ASSESSMENT/ REPORT/dinpreport046.pdf

Fennell, T. R., Krol, W. L., Sumner, S. C., and Snyder, R. W. (2004). Pharmacokinetics of dibutylphthalate in pregnant rats. *Toxicol. Sci.* 82, 407-418.

Fernandez-Salguero, P. M., Ward, J. M., Sundberg, J. P., and Gonzalez, F. J. (1997). Lesions of aryl-hydrocarbon receptor-deficient mice. *Vet. Pathol.* 34, 605-614.

Ferrara, D., Hallmark, N., Scott, H., Brown, R., McKinnell, C., Mahood, I. K., and Sharpe, R. M. (2006). Acute and long-term effects of *in utero* exposure of rats to di(*n*-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology.* 147, 5352-5362.

Ferreira, F. M., Madeira, V. M., and Moreno, A. J. (1997). Interactions of 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene with mitochondrial oxidative phosphorylation. *Biochem. Pharmacol.* 53, 299-308.

Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kowk, G., Weller, P. A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I. D., and Goodfellow, P. N. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature.* 372, 525-530.

Freyberger, A., Ellinger-Ziegelbauer, H., and Krotlinger, F. (2007). Evaluation of the rodent Hershberger bioassay: testing of coded chemicals and supplementary molecular-biological and biochemical investigations. *Toxicology.* 239, 77-88.

Fukuzawa, N. H., Ohsako, S., Wu, Q., Sakaue, M., Fujii-Kuriyama, Y., Baba, T., and Tohyama, C. (2004). Testicular cytochrome P450scc and LHR as possible

targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the mouse. *Mol. Cell. Endocrinol.* 221, 87-96.

Garabrant, D. H., Held, J., Langholz, B., Peters, J. M., and Mack, T. M. (1992). DDT and related compounds and risk of pancreatic cancer. *J. Natl. Cancer Inst.* 84, 764-771.

Gill, W. B., Schumacher, G. F., and Bibbo, M. (1976). Structural and functional abnormalities in the sex organs of male offspring of mothers treated with diethylstilbestrol (DES). *J. Reprod. Med.* 16, 147-153.

Gill, W. B., Schumacher, G. F., Bibbo, M., Straus, F. H.,2nd, and Schoenberg, H. W. (1979). Association of diethylstilbestrol exposure *in utero* with cryptorchidism, testicular hypoplasia and semen abnormalities. *J. Urol.* 122, 36-39.

Giusti, R. M., Iwamoto, K., and Hatch, E. E. (1995). Diethylstilbestrol revisited: a review of the long-term health effects. *Ann. Intern. Med.* 122, 778-788.

Gonzalez, R. J., Tamm, E. P., Ng, C., Phan, A. T., Vassilopoulou-Sellin, R., Perrier, N. D., Evans, D. B., and Lee, J. E. (2007). Response to mitotane predicts outcome in patients with recurrent adrenal cortical carcinoma. *Surgery.* 142, 867-75; discussion 867-75.

Goodrich, L. V. and Scott, M. P. (1998). Hedgehog and patched in neural development and disease. *Neuron.* 21, 1243-1257.

Goto, K., Koizumi, K., Takaori, H., Fujii, Y., Furuyama, Y., Saika, O., Suzuki, H., Saito, K., and Suzuki, K. (2004). Effects of flutamide on sex maturation and behavior of offspring born to female rats treated during late pregnancy. *J. Toxicol. Sci.* 29, 517-534.

Gray, L. E.,Jr, Kelce, W. R., Monosson, E., Ostby, J. S., and Birnbaum, L. S. (1995). Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. *Toxicol. Appl. Pharmacol.* 131, 108-118.

Gray, L. E.,Jr, Ostby, J., Furr, J., Price, M., Veeramachaneni, D. N., and Parks, L. (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol. Sci.* 58, 350-365.

Gray, L. E.,Jr, Ostby, J., Wilson, V., Lambright, C., Bobseine, K., Hartig, P., Hotchkiss, A., Wolf, C., Furr, J., Price, M., Parks, L., Cooper, R. L., Stoker, T. E., Laws, S. C., Degitz, S. J., Jensen, K. M., Kahl, M. D., Korte, J. J., Makynen, E. A., Tietge, J. E., and Ankley, G.T. (2002). Xenoendocrine disrupters-tiered screening and testing: filling key data gaps. *Toxicology.* 181-182, 371-382.

Grino, M., Paulmyer-Lacroix, O., Anglade, G., and Oliver, C. (1995). Molecular aspects of the regulation of the hypothalamo-pituitary-adrenal axis during development in the rat. *Ann. N. Y. Acad. Sci.* 771, 339-351.

Guillette, L. J.,Jr, Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., and Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* 102, 680-688.

Guillette, L. J.,Jr and Gunderson, M. P. (2001). Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction.* 122, 857-864.

Guyot, R., Odet, F., Leduque, P., Forest, M. G., and Le Magueresse-Battistoni, B. (2004). Diethylstilbestrol inhibits the expression of the steroidogenic acute regulatory protein in mouse fetal testis. *Mol. Cell. Endocrinol.* 220, 67-75.

Haavisto, A. M., Pettersson, K., Bergendahl, M., Perheentupa, A., Roser, J. F., and Huhtaniemi, I. (1993). A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology.* 132, 1687-1691.

Haavisto, T. E., Adamsson, N. A., Myllymaki, S. A., Toppari, J., and Paranko, J. (2003). Effects of 4-tert-octylphenol, 4-*tert*-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat. *Reprod. Toxicol.* 17, 593-605.

Habert, R., Lejeune, H., and Saez, J. M. (2001). Origin, differentiation and regulation of fetal and adult Leydig cells. *Mol. Cell. Endocrinol.* 179, 47-74.

Habert, R. and Picon, R. (1982). Control of testicular steroidogenesis in foetal rat: effect of decapitation on testosterone and plasma luteinizing hormone-like activity. *Acta Endocrinol. (Copenh).* 99, 466-473.

Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development.* 121, 1603-1614.

Hammer, G. D., Parker, K. L., and Schimmer, B. P. (2005). Minireview: transcriptional regulation of adrenocortical development. *Endocrinology.* 146, 1018-1024.

Hass, U., Scholze, M., Christiansen, S., Dalgaard, M., Vinggaard, A. M., Axelstad, M., Metzdorff, S. B., and Kortenkamp, A. (2007). Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environ. Health Perspect.* 115 Suppl 1, 122-128.

Hatano, O., Takakusu, A., Nomura, M., and Morohashi, K. (1996). Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes Cells.* 1, 663-671.

Henck, J. M., New, M. A., Kociba, R. J., and Rao, K. S. (1981). 2,3,7,8-Tetrachlorodibenzo-*P*-Dioxin: Acute Oral Toxicity in Hamsters. *Toxicol. Appl. Pharmacol.* 59, 405-407.

Herbst, A. L. (1976). Summary of the changes in the human female genital tract as a consequence of maternal diethylstilbestrol therapy. *J. Toxicol. Environ. Health Suppl.* 1, 13-20.

Herbst, A. L., Poskanzer, D. C., Robboy, S. J., Friedlander, L.,and Scully, R.E. (1975). Prenatal exposure to stilbestrol. A prospective comparison of exposed female offspring with unexposed controls. *N. Engl. J. Med.* 292, 334-339.

Hickey, J. J. and Anderson, D. W. (1968). Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. *Science.* 162, 271-273.

Ho, M.M. and Vinson, G.P. (1993). 11 beta-Hydroxylase gene expression in the rat adrenal cortex. *J. Endocrinol.* 139, 301-306.

Hotchkiss, A. K., Rider, C. V., Blystone, C. R., Wilson, V. S., Hartig, P. C., Ankley, G. T., Foster, P. M., Gray, C. L., and Gray, L. E. (2008). Fifteen years after "Wingspread"-environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol. Sci.* 105, 235-259.

Hovmoller, M. L., Osuna, A., Eklof, O., Fredga, K., Hjerpe, A., Linsten, J., Ritzen, M., Stanescu, V., and Svenningsen, N. (1977). Camptomelic dwarfism. A genetically determined mesenchymal disorder combined with sex reversal. *Hereditas.* 86, 51-62.

Huhtaniemi, I. and Pelliniemi, L. J. (1992). Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proc. Soc. Exp. Biol. Med.* 201, 125-140.

Hunter, D. J., Hankinson, S. E., Laden, F., Colditz, G. A., Manson, J. E., Willett, W. C., Speizer, F. E., and Wolff, M. S. (1997). Plasma organochlorine levels and the risk of breast cancer. *N. Engl. J. Med.* 337, 1253-1258.

Ikeda, Y., Tanaka, H., and Esaki, M. (2008). Effects of gestational diethylstilbestrol treatment on male and female gonads during early embryonic development. *Endocrinology.* 149, 3970-3979.

Imperato-McGinley, J., Sanchez, R. S., Spencer, J. R., Yee, B., and Vaughan, E. D. (1992). Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology.* 131, 1149-1156.

Imperato-McGinley, J. and Zhu, Y. S. (2002). Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency. *Mol. Cell. Endocrinol.* 198, 51-59.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* 15, 3059-3087.

International Programme on Chemical Safety. http://whqlibdoc.who.int/hq/2002/a76784_ch1.pdf.

Jamieson, R. V., Zhou, S. X., Wheatley, S. C., Koopman, P., and Tam, P. P. (1998). Sertoli cell differentiation and Y-chromosome activity: a developmental study of X-linked transgene activity in sex-reversed X/XSxra mouse embryos. *Dev. Biol.* 199, 235-244.

Jenkins, E. P., Andersson, S., Imperato-McGinley, J., Wilson, J. D., and Russell, D. W. (1992). Genetic and pharmacological evidence for more than one human steroid 5 alpha-reductase. *J. Clin. Invest.* 89, 293-300.

Joensen, U. N., Jorgensen, N., Rajpert-De Meyts, E., and Skakkebaek, N. E. (2008). Testicular dysgenesis syndrome and Leydig cell function. *Basic Clin. Pharmacol. Toxicol.* 102, 155-161.

Joffe, M. (2001). Are problems with male reproductive health caused by endocrine disruption? *Occup. Environ. Med.* 58, 281-7; quiz 287-8, 260.

Johnson, L., Wilker, C. E., Safe, S. H., Scott, B., Dean, D. D., and White, P. H. (1994). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin reduces the number, size, and organelle content of Leydig cells in adult rat testes. *Toxicology.* 89, 49-65.

Johnston, H., Baker, P. J., Abel, M., Charlton, H. M., Jackson, G., Fleming, L., Kumar, T. R., and O'Shaughnessy, P. J. (2004). Regulation of Sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse. *Endocrinology.* 145, 318-329.

Jonsson, C. J., Rodriguez-Martinez, H., Lund, B. O., Bergman, A., and Brandt, I. (1991). Adrenocortical toxicity of 3-methylsulfonyl-DDE in mice. II. Mitochondrial changes following ecologically relevant doses. *Fundam. Appl. Toxicol.* 16, 365-374.

Jonsson, J., Rodriguez-Martinez, H., and Brandt, I. (1995). Transplacental toxicity of 3-methylsulphonyl-DDE in the developing adrenal cortex in mice. *Reprod. Toxicol.* 9, 257-264.

Jost, A., Magre, S., and Agelopoulou, R. (1981). Early stages of testicular differentiation in the rat. *Hum. Genet.* 58, 59-63.

Kalderon, D. (2000). Transducing the hedgehog signal. *Cell.* 103, 371-374.

Kassim, N. M., McDonald, S. W., Reid, O., Bennett, N. K., Gilmore, D. P., and Payne, A. P. (1997). The effects of pre- and postnatal exposure to the nonsteroidal antiandrogen flutamide on testis descent and morphology in the Albino Swiss rat. *J. Anat.* 190 (Pt 4), 577-588.

Kavlock, R., Boekelheide, K., Chapin, R., Cunningham, M., Faustman, E., Foster, P., Golub, M., Henderson, R., Hinberg, I., Little, R., Seed, J., Shea, K., Tabacova, S., Tyl, R., Williams, P., and Zacharewski, T. (2002). NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-isononyl phthalate. *Reprod. Toxicol.* 16, 679-708.

Keegan, C. E. and Hammer, G. D. (2002). Recent insights into organogenesis of the adrenal cortex. *Trends Endocrinol. Metab.* 13, 200-208.

Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A., and Wilson, E. M. (1995). Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature.* 375, 581-585.

Kempna, P. and Fluck, C. E. (2008). Adrenal gland development and defects. *Best Pract. Res. Clin. Endocrinol. Metab.* 22, 77-93.

Kendall, S. K., Samuelson, L. C., Saunders, T. L., Wood, R. I., and Camper, S. A. (1995). Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadal and hypothyroid mice. *Genes Dev.* 9, 2007-2019.

Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996). A male-specific role for SOX9 in vertebrate sex determination. *Development.* 122, 2813-2822.

Kerr, J. B. and Knell, C. M. (1988). The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. *Development.* 103, 535-544.

Kiiveri, S., Liu, J., Westerholm-Ormio, M., Narita, N., Wilson, D. B., Voutilainen, R., and Heikinheimo, M. (2002). Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. *Endocrinology.* 143, 3136-3143.

Kiviranta, H., Hallikainen, A., Ovaskainen, M. L., Kumpulainen, J., and Vartiainen, T. (2001). Dietary intakes of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and polychlorinated biphenyls in Finland. *Food Addit. Contam.* 18, 945-953.

Klotz, L. (2008). Maximal androgen blockade for advanced prostate cancer. *Best Pract. Res. Clin. Endocrinol. Metab.* 22, 331-340.

Koopman, P., Munsterberg, A., Capel, B., Vivian, N., and Lovell-Badge, R. (1990). Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature.* 348, 450-452.

Korkalainen, M., Tuomisto, J., and Pohjanvirta, R. (2004). Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) of aryl hydrocarbon receptor repressor in a TCDD-sensitive and a TCDD-resistant rat strain. *Biochem. Biophys. Res. Commun.* 315, 123-131.

Kortenkamp, A. (2008). Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. *Int. J. Androl.* 31, 233-240.

Krishnan, V., Wang, X., and Safe, S. (1994). Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *J. Biol. Chem.* 269, 15912-15917.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology.* 138, 863-870.

Kulkarni, P. S., Crespo, J. G., and Afonso, C. A. (2008). Dioxins sources and current remediation technologies--a review. *Environ. Int.* 34, 139-153.

Le Goascogne, C., Sananes, N., Gouezou, M., Takemori, S., Kominami, S., Baulieu, E. E., and Robel, P. (1991). Immunoreactive cytochrome P-450(17 alpha) in rat and guinea-pig gonads, adrenal glands and brain. *J. Reprod. Fertil.* 93, 609-622.

Leers-Sucheta, S., Morohashi, K., Mason, J. I., and Melner, M. H. (1997). Synergistic activation of the human type II 3beta-hydroxysteroid dehydrogenase /delta5-delta4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. *J. Biol. Chem.* 272, 7960-7967.

Li, L. H., Jester, W. F.,Jr, and Orth, J. M. (1998). Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. *Toxicol. Appl. Pharmacol.* 153, 258-265.

Li, S., Hansman, R., Newbold, R., Davis, B., McLachlan, J. A., and Barrett, J. C. (2003). Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. *Mol. Carcinog.* 38, 78-84.

Li, S., Washburn, K. A., Moore, R., Uno, T., Teng, C., Newbold, R. R., McLachlan, J. A., and Negishi, M. (1997). Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. *Cancer Res.* 57, 4356-4359.

Li, X., Weber, L. W., and Rozman, K. K. (1995). Toxicokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female Sprague-Dawley rats including placental and lactational transfer to fetuses and neonates. *Fundam. Appl. Toxicol.* 27, 70-76.

Liggins, G. C. (1994). The role of cortisol in preparing the fetus for birth. *Reprod. Fertil. Dev.* 6, 141-150.

Lin, D., Sugawara, T., Strauss, J. F.,3rd, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995). Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science.* 267, 1828-1831.

Lindstrom, V., Brandt, I., and Lindhe, O. (2008). Species differences in 3-methylsulphonyl-DDE bioactivation by adrenocortical tissue. *Arch. Toxicol.* 82, 159-163.

Liu, K., Lehmann, K. P., Sar, M., Young, S. S., and Gaido, K. W. (2005). Gene expression profiling following *in utero* exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. *Biol. Reprod.* 73, 180-192.

Longnecker, M. P., Rogan, W. J., and Lucier, G. (1997). The human health effects of DDT (dichlorodiphenyltrichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. *Annu. Rev. Public Health.* 18, 211-244.

Lund, B. O. and Lund, J. (1995). Novel involvement of a mitochondrial steroid hydroxylase (P450c11) in xenobiotic metabolism. *J. Biol. Chem.* 270, 20895-20897.

Luo, X., Ikeda, Y., and Parker, K. L. (1995). The cell-specific nuclear receptor steroidogenic factor 1 plays multiple roles in reproductive function. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 350, 279-283.

Mably, T. A., Bjerke, D. L., Moore, R. W., Gendron-Fitzpatrick, A., and Peterson, R. E. (1992a). *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 3. Effects on spermatogenesis and reproductive capability. *Toxicol. Appl. Pharmacol.* 114, 118-126.

Mably, T. A., Moore, R. W., and Peterson, R. E. (1992b). *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 1. Effects on androgenic status. *Toxicol. Appl. Pharmacol.* 114, 97-107.

Mackenzie, B. R., Almesjo, L., and Hansson, S. (2004). Fish, fishing, and pollutant reduction in the Baltic Sea. *Environ. Sci. Technol.* 38, 1970-1976.

Magre, S. and Jost, A. (1991). Sertoli cells and testicular differentiation in the rat fetus. *J. Electron Microsc. Tech.* 19, 172-188.

Majdic, G., Millar, M. R., and Saunders, P. T. (1995). Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *J. Endocrinol.* 147, 285-293.

Majdic, G., Sharpe, R. M., O'Shaughnessy, P. J., and Saunders, P. T. (1996). Expression of cytochrome P450 17alpha-hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology.* 137, 1063-1070.

Matlai, P. and Beral, V. (1985). Trends in congenital malformations of external genitalia. *Lancet.* 1, 108.

McConnell, E. E., Moore, J. A., Haseman, J. K., and Harris, M. W. (1978). The comparative toxicity of chlorinated dibenzo-*p*-dioxins in mice and guinea pigs. *Toxicol. Appl. Pharmacol.* 44, 335-356.

McIntyre, B. S., Barlow, N. J., and Foster, P. M. (2001). Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol. Sci.* 62, 236-249.

McKee, R. H., El-Hawari, M., Stoltz, M., Pallas, F., and Lington, A. W. (2002). Absorption, disposition and metabolism of di-isononyl phthalate (DINP) in F-344 rats. *J. Appl. Toxicol.* 22, 293-302.

McKinnell, C., Atanassova, N., Williams, K., Fisher, J. S., Walker, M., Turner, K. J., Saunders, T. K., and Sharpe, R. M. (2001). Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *J. Androl.* 22, 323-338.

Miller, C., Degenhardt, K., and Sassoon, D. A. (1998). Fetal exposure to DES results in de-regulation of Wnt7a during uterine morphogenesis. *Nat. Genet.* 20, 228-230.

Miller, W. L., Auchus, R. J., and Geller, D. H. (1997). The regulation of 17,20 lyase activity. *Steroids.* 62, 133-142.

Milligan, S. R., Khan, O., and Nash, M. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steroid binding proteins in human and rainbow trout (Oncorhynchus mykiss) plasma. *Gen. Comp. Endocrinol.* 112, 89-95.

Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999). Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev.* 13, 20-25.

Mimura, J. and Fujii-Kuriyama, Y. (2003). Functional role of AhR in the expression of toxic effects by TCDD. *Biochim. Biophys. Acta.* 1619, 263-268.

Mitani, F., Mukai, K., Miyamoto, H., Suematsu, M., and Ishimura, Y. (1999). Development of functional zonation in the rat adrenal cortex. *Endocrinology.* 140, 3342-3353.

Mitani, F., Suzuki, H., Hata, J., Ogishima, T., Shimada, H., and Ishimura, Y. (1994). A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology.* 135, 431-438.

Miyata, K., Yabushita, S., Sukata, T., Sano, M., Yoshino, H., Nakanishi, T., Okuno, Y., and Matsuo, M. (2002). Effects of perinatal exposure to flutamide on sex hormones and androgen-dependent organs in F1 male rats. *J. Toxicol. Sci.* 27, 19-33.

Mocarelli, P., Gerthoux, P. M., Patterson, D. G.,Jr, Milani, S., Limonta, G., Bertona, M., Signorini, S., Tramacere, P., Colombo, L., Crespi, C., Brambilla, P., Sarto, C., Carreri, V., Sampson, E. J., Turner, W. E., and Needham, L. L. (2008). Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environ. Health Perspect.* 116, 70-77.

Moller, H. (1998). Trends in sex-ratio, testicular cancer and male reproductive hazards: are they connected? *APMIS.* 106, 232-8; discussion 238-9.

Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., and Lovell-Badge, R. (1996). Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat. Genet.* 14, 62-68.

Morgan, D. P. and Roan, C. C. (1971). Absorption, storage, and metabolic conversion of ingested DDT and DDT metabolites in man. *Arch. Environ. Health.* 22, 301-308.

Morohashi, K. (1997). The ontogenesis of the steroidogenic tissues. *Genes Cells.* 2, 95-106.

Morohashi, K., Honda, S., Inomata, Y., Handa, H., and Omura, T. (1992). A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J. Biol. Chem.* 267, 17913-17919.

Munsterberg, A. and Lovell-Badge, R. (1991). Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. *Development.* 113, 613-624.

Mutoh, J., Taketoh, J., Okamura, K., Kagawa, T., Ishida, T., Ishii, Y., and Yamada, H. (2006). Fetal pituitary gonadotropin as an initial target of dioxin in its impairment of cholesterol transportation and steroidogenesis in rats. *Endocrinology.* 147, 927-936.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* 156, 81-95.

Mylchreest, E., Sar, M., Wallace, D. G., and Foster, P. M. (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. *Reprod. Toxicol.* 16, 19-28.

Myllymaki, S. A., Haavisto, T. E., Brokken, L. J., Viluksela, M., Toppari, J., and Paranko, J. (2005a). In utero and lactational exposure to TCDD; steroidogenic outcomes differ in male and female rat pups. *Toxicol. Sci.* 88, 534-544.

Myllymaki, S. A., Karjalainen, M., Haavisto, T. E., Toppari, J., and Paranko, J. (2005b). Infantile 4-*tert*-octylphenol exposure transiently inhibits rat ovarian steroidogenesis and steroidogenic acute regulatory protein (StAR) expression. *Toxicol. Appl. Pharmacol.* 207, 59-68.

Nef, S. and Parada, L. F. (1999). Cryptorchidism in mice mutant for Insl3. *Nat. Genet.* 22, 295-299.

Newbold, R. R. (2004). Lessons learned from perinatal exposure to diethylstilbestrol. *Toxicol. Appl. Pharmacol.* 199, 142-150.

Noguchi, J., Hikono, H., Sato, S., Watanabe, G., Taya, K., Sasamoto, S., and Hasegawa, Y. (1997). Ontogeny of inhibin secretion in the rat testis: secretion of inhibin-related proteins from fetal Leydig cells and of bioactive inhibin from Sertoli cells. *J. Endocrinol.* 155, 27-34.

Norgil Damgaard, I., Main, K. M., Toppari, J., and Skakkebaek, N. E. (2002). Impact of exposure to endocrine disrupters *in utero* and in childhood on adult reproduction. *Best Pract. Res. Clin. Endocrinol. Metab.* 16, 289-309.

Normington, K. and Russell, D. W. (1992). Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. *J. Biol. Chem.* 267, 19548-19554.

Ogishima, T., Suzuki, H., Hata, J., Mitani, F., and Ishimura, Y. (1992). Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011 beta in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology.* 130, 2971-2977.

Ohsako, S., Miyabara, Y., Nishimura, N., Kurosawa, S., Sakaue, M., Ishimura, R., Sato, M., Takeda, K., Aoki, Y., Sone, H., Tohyama, C., and Yonemoto, J. (2001). Maternal exposure to a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppressed the development of reproductive organs of male rats: dose-dependent increase of mRNA levels of 5alpha-reductase type 2 in contrast to decrease of androgen receptor in the pubertal ventral prostate. *Toxicol. Sci.* 60, 132-143.

Ohsako, S., Miyabara, Y., Sakaue, M., Ishimura, R., Kakeyama, M., Izumi, H., Yonemoto, J., and Tohyama, C. (2002). Developmental stage-specific effects of perinatal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on reproductive organs of male rat offspring. *Toxicol. Sci.* 66, 283-292.

Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., Takahashi, S., Kouzmenko, A., Nohara, K., Chiba, T., Fujii-Kuriyama, Y., and Kato, S. (2007). Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature.* 446, 562-566.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., and Kato, S. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature.* 423, 545-550.

Olson, J. R., Holscher, M. A., and Neal, R. A. (1980). Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the golden Syrian hamster. *Toxicol. Appl. Pharmacol.* 55, 67-78.

Omezzine, A., Chater, S., Mauduit, C., Florin, A., Tabone, E., Chuzel, F., Bars, R., and Benahmed, M. (2003). Long-term apoptotic cell death process with increased expression and activation of caspase-3 and -6 in adult rat germ cells exposed in utero to flutamide. *Endocrinology.* 144, 648-661.

Orth, D. N. (1995). Cushing's syndrome. *N. Engl. J. Med.* 332, 791-803.

Orth, J. M. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat. Rec.* 203, 485-492.

Orth, J. M., Gunsalus, G. L., and Lamperti, A. A. (1988). Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology.* 122, 787-794.

O'Shaughnessy, P. J., Baker, P., Sohnius, U., Haavisto, A. M., Charlton, H. M., and Huhtaniemi, I. (1998). Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology.* 139, 1141-1146.

O'Shaughnessy, P. J., Fleming, L. M., Jackson, G., Hochgeschwender, U., Reed, P., and Baker, P. J. (2003). Adrenocorticotropic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. *Endocrinology.* 144, 3279-3284.

Pakarinen, P., Proshlyakova, E., and Huhtaniemi, I. (1994). Pituitary-gonadal interactions in perinatal rats: relationships of plasma luteinizing hormone and testosterone concentrations, and pituitary levels of LH subunit mRNAs. *Neuroendocrinology.* 60, 42-49.

Palmlund, I. (1996). Exposure to a xenoestrogen before birth: the diethylstilbestrol experience. *J. Psychosom. Obstet. Gynaecol.* 17, 71-84.

Park, S. Y., Tong, M., and Jameson, J. L. (2007). Distinct roles for steroidogenic factor 1 and desert hedgehog pathways in fetal and adult Leydig cell development. *Endocrinology.* 148, 3704-3710.

Parker, K. L. and Schimmer, B. P. (2002). Genes essential for early events in gonadal development. *Ann. Med.* 34, 171-178.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., Klinefelter, G. R., Barlow, N. J., and Gray, L. E.,Jr. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.* 58, 339-349.

Pasca di Magliano, M. and Hebrok, M. (2003). Hedgehog signalling in cancer formation and maintenance. *Nat. Rev. Cancer.* 3, 903-911.

Pelclova, D., Urban, P., Preiss, J., Lukas, E., Fenclova, Z., Navratil, T., Dubska, Z., and Senholdova, Z.

(2006). Adverse health effects in humans exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Rev. Environ. Health.* 21, 119-138.

Perkins, L. M. and Payne, A. H. (1988). Quantification of P450scc, P450(17) alpha, and iron sulfur protein reductase in Leydig cells and adrenals of inbred strains of mice. *Endocrinology.* 123, 2675-2682.

Peterson, R. E., Theobald, H. M., and Kimmel, G. L. (1993). Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. *Crit. Rev. Toxicol.* 23, 283-335.

Petrulis, J. R. and Perdew, G. H. (2002). The role of chaperone proteins in the aryl hydrocarbon receptor core complex. *Chem. Biol. Interact.* 141, 25-40.

Pohjanvirta, R. and Tuomisto, J. (1994). Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals: effects, mechanisms, and animal models. *Pharmacol. Rev.* 46, 483-549.

Pohjanvirta, R., Unkila, M., and Tuomisto, J. (1993). Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. *Pharmacol. Toxicol.* 73, 52-56.

Pohjanvirta, R., Viluksela, M., Tuomisto, J. T., Unkila, M., Karasinska, J., Franc, M. A., Holowenko, M., Giannone, J. V., Harper, P. A., Tuomisto, J., and Okey, A. B. (1999). Physicochemical differences in the AH receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. *Toxicol. Appl. Pharmacol.* 155, 82-95.

Pohjanvirta, R., Wong, J. M., Li, W., Harper, P. A., Tuomisto, J., and Okey, A. B. (1998). Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant rat strain. *Mol. Pharmacol.* 54, 86-93.

Poland, A. and Glover, E. (1980). 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin: segregation of toxocity with the Ah locus. *Mol. Pharmacol.* 17, 86-94.

Reinhart, A. J., Williams, S. C., Clark, B. J., and Stocco, D. M. (1999). SF-1 (steroidogenic factor-1) and C/EBP beta (CCAAT/enhancer binding protein-beta) cooperate to regulate the murine StAR (steroidogenic acute regulatory) promoter. *Mol. Endocrinol.* 13, 729-741.

Rey, R., Lukas-Croisier, C., Lasala, C., and Bedecarras, P. (2003). AMH/MIS: what we know already about the gene, the protein and its regulation. *Mol. Cell. Endocrinol.* 211, 21-31.

Rider, C. V., Furr, J., Wilson, V. S., and Gray, L. E.,Jr. (2008). A mixture of seven antiandrogens induces reproductive malformations in rats. *Int. J. Androl.* 31, 249-262.

Rivas, A., Fisher, J. S., McKinnell, C., Atanassova, N., and Sharpe, R. M. (2002a). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. *Endocrinology.* 143, 4797-4808.

Rivas, A., Fisher, J. S., McKinnell, C., Atanassova, N., and Sharpe, R. M. (2002b). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. *Endocrinology.* 143, 4797-4808.

Robertson, K. D. and Jones, P. A. (2000). DNA methylation: past, present and future directions. *Carcinogenesis.* 21, 461-467.

Rogan, W. J. and Chen, A. (2005). Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). *Lancet*. 366, 763-773.

Roman, B. L., Pollenz, R. S., and Peterson, R. E. (1998). Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah receptor down-regulation. *Toxicol. Appl. Pharmacol.* 150, 228-239.

Roman, B. L., Sommer, R. J., Shinomiya, K., and Peterson, R. E. (1995). In utero and lactational exposure of the male rat to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: impaired prostate growth and development without inhibited androgen production. *Toxicol. Appl. Pharmacol.* 134, 241-250.

Ruiz i Altaba, A., Sanchez, P., and Dahmane, N. (2002). Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat. Rev. Cancer.* 2, 361-372.

Sadasivaiah, S., Tozan, Y., and Breman, J. G. (2007). Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? *Am. J. Trop. Med. Hyg.* 77, 249-263.

Saez, J. M. (1994). Leydig cells: endocrine, paracrine, and autocrine regulation. *Endocr. Rev.* 15, 574-626.

Salisbury, R. L., Dudley, S. D., and Weisz, J. (1982). Effect of gonadotrophin-releasing hormone on circulating levels of immunoreactive luteinizing hormone in fetal rats. *Neuroendocrinology.* 35, 265-269.

Sassoon, D. (1999). Wnt genes and endocrine disruption of the female reproductive tract: a genetic approach. *Mol. Cell. Endocrinol.* 158, 1-5.

Saunders, P. T., Majdic, G., Parte, P., Millar, M. R., Fisher, J. S., Turner, K. J., and Sharpe, R. M. (1997). Fetal and perinatal influence of xenoestrogens on testis gene expression. *Adv. Exp. Med. Biol.* 424, 99-110.

Savu, L., Benassayag, C., Vallette, G., Christeff, N., and Nunez, E. (1981). Mouse alpha 1-fetoprotein and albumin. A comparison of their binding properties with estrogen and fatty acid ligands. *J. Biol. Chem.* 256, 9414-9418.

Schafer, A. J., Foster, J. W., Kwok, C., Weller, P. A., Guioli, S., and Goodfellow, P. N. (1996). Campomelic dysplasia with XY sex reversal: diverse phenotypes resulting from mutations in a single gene. *Ann. N. Y. Acad. Sci.* 785, 137-149.

Schmahl, J., Eicher, E. M., Washburn, L. L., and Capel, B. (2000). Sry induces cell proliferation in the mouse gonad. *Development.* 127, 65-73.

Schneider, R. A., Hu, D., and Helms, J. A. (1999). From head to toe: conservation of molecular signals regulating limb and craniofacial morphogenesis. *Cell Tissue Res.* 296, 103-109.

Schteingart, D. E., Motazedi, A., Noonan, R. A., and Thompson, N. W. (1982). Treatment of adrenal carcinomas. *Arch. Surg.* 117, 1142-1146.

Schultz, R., Suominen, J., Varre, T., Hakovirta, H., Parvinen, M., Toppari, J., and Pelto-Huikko, M. (2003). Expression of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator messenger ribonucleic acids and proteins in rat and human testis. *Endocrinology.* 144, 767-776.

Schwetz, B. A., Norris, J. M., Sparschu, G. L., Rowe, U. K., Gehring, P. J., Emerson, J. L., and Gerbig, C. G. (1973). Toxicology of chlorinated dibenzo-*p*-dioxins. *Environ. Health Perspect.* 5, 87-99.

Scott, H. M., Hutchison, G. R., Jobling, M. S., McKinnell, C., Drake, A. J., and Sharpe, R. M. (2008). Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat. *Endocrinology.* 149, 5280-5287.

Scott, H. M., Hutchison, G. R., Mahood, I. K., Hallmark, N., Welsh, M., De Gendt, K., Verhoeven, G., O'Shaughnessy, P., and Sharpe, R. M. (2007). Role of androgens in fetal testis development and dysgenesis. *Endocrinology.* 148, 2027-2036.

Sharpe, R. M. (2001). Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol. Lett.* 120, 221-232.

Sharpe, R. M., McKinnell, C., Kivlin, C., and Fisher, J. S. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction.* 125, 769-784.

Sheehan, D. M. and Young, M. (1979). Diethylstilbestrol and estradiol binding to serum albumin and pregnancy plasma of rat and human. *Endocrinology.* 104, 1442-1446.

Simanainen, U., Adamsson, A., Tuomisto, J. T., Miettinen, H. M., Toppari, J., Tuomisto, J., and Viluksela, M. (2004a). Adult 2,3,7,8-tetrachlorodi benzo-*p*-dioxin (TCDD) exposure and effects on male reproductive organs in three differentially TCDD-susceptible rat lines. *Toxicol. Sci.* 81, 401-407.

Simanainen, U., Haavisto, T., Tuomisto, J. T., Paranko, J., Toppari, J., Tuomisto, J., Peterson, R. E., and Viluksela, M. (2004b). Pattern of male reproductive system effects after in utero and lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in three differentially TCDD-sensitive rat lines. *Toxicol. Sci.* 80, 101-108.

Simard, J., Couet, J., Durocher, F., Labrie, Y., Sanchez, R., Breton, N., Turgeon, C., and Labrie, F. (1993). Structure and tissue-specific expression of a novel member of the rat 3 beta-hydroxysteroid dehydrogenase/ delta 5-delta 4 isomerase (3 beta-HSD) family. The exclusive 3 beta-HSD gene expression in the skin. *J. Biol. Chem.* 268, 19659-19668.

Simard, J., Ricketts, M. L., Gingras, S., Soucy, P., Feltus, F. A., and Melner, M. H. (2005). Molecular biology of the 3 beta-hydroxysteroid dehydrogenase/ delta5-delta4 isomerase gene family. *Endocr. Rev.* 26, 525-582.

Simpson, E. R. and Boyd, G. S. (1966). The cholesterol side-chain cleavage system of the adrenal cortex: a mixed-function oxidase. *Biochem. Biophys. Res. Commun.* 24, 10-17.

Skakkebaek, N. E., Rajpert-De Meyts, E., and Main, K. M. (2001). Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum. Reprod.* 16, 972-978.

Stocco, D. M. (2000). Intramitochondrial cholesterol transfer. *Biochim. Biophys. Acta.* 1486, 184-197.

Stocco, D. M. and Clark, B. J. (1996). Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem. Pharmacol.* 51, 197-205.

Suzuki, A., Urushitani, H., Sato, T., Kobayashi, T., Watanabe, H., Ohta, Y., and Iguchi, T. (2007). Gene expression change in the Mullerian duct of the mouse fetus exposed to diethylstilbestrol in utero. *Exp. Biol. Med. (Maywood).* 232, 503-514.

Swain, A., Narvaez, V., Burgoyne, P., Camerino, G., and Lovell-Badge, R. (1998). Dax1 antagonizes Sry action in mammalian sex determination. *Nature.* 391, 761-767.

Swain, A., Zanaria, E., Hacker, A., Lovell-Badge, R., and Camerino, G. (1996). Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nat. Genet.* 12, 404-409.

Szczepny, A., Hime, G. R., and Loveland, K. L. (2006). Expression of hedgehog signalling components in adult mouse testis. *Dev. Dyn.* 235, 3063-3070.

Tan, K. A., De Gendt, K., Atanassova, N., Walker, M., Sharpe, R. M., Saunders, P. T., Denolet, E., and Verhoeven, G. (2005). The role of androgens in sertoli cell proliferation and functional maturation: studies in mice with total or Sertoli cell-selective ablation of the androgen receptor. *Endocrinology.* 146, 2674-2683.

Tapanainen, J., Kuopio, T., Pelliniemi, L. J., and Huhtaniemi, I. (1984). Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol. Reprod.* 31, 1027-1035.

Taylor, H. S. (2000). The role of HOX genes in the development and function of the female reproductive tract. *Semin. Reprod. Med.* 18, 81-89.

Tevosian, S. G., Albrecht, K. H., Crispino, J. D., Fujiwara, Y., Eicher, E. M., and Orkin, S. H. (2002). Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development.* 129, 4627-4634.

Theobald, H. M., Roman, B. L., Lin, T. M., Ohtani, S., Chen, S. W., and Peterson, R. E. (2000). 2,3,7,8-Tetrachlorodibenzo-*P*-Dioxin Inhibits Luminal Cell Differentiation and Androgen Responsiveness of the Ventral Prostate without Inhibiting Prostatic 5alpha-Dihydrotestosterone Formation Or Testicular

Androgen Production in Rat Offspring. *Toxicol. Sci.* 58, 324-338.

Thompson, C. J., Ross, S. M., and Gaido, K. W. (2004). Di(*n*-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. *Endocrinology.* 145, 1227-1237.

Tian, Y., Ke, S., Thomas, T., Meeker, R. J., and Gallo, M. A. (1998). Transcriptional suppression of estrogen receptor gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J. Steroid Biochem. Mol. Biol.* 67, 17-24.

Toyoda, K., Shibutani, M., Tamura, T., Koujitani, T., Uneyama, C., and Hirose, M. (2000). Repeated dose (28 days) oral toxicity study of flutamide in rats, based on the draft protocol for the 'Enhanced OECD Test Guideline 407' for screening for endocrine-disrupting chemicals. *Arch. Toxicol.* 74, 127-132.

Tremblay, J. J. and Viger, R. S. (2003). Novel roles for GATA transcription factors in the regulation of steroidogenesis. *J. Steroid Biochem. Mol. Biol.* 85, 291-298.

Tuomisto, J. T., Viluksela, M., Pohjanvirta, R., and Tuomisto, J. (1999). The AH receptor and a novel gene determine acute toxic responses to TCDD: segregation of the resistant alleles to different rat lines. *Toxicol. Appl. Pharmacol.* 155, 71-81.

Turusov, V., Rakitsky, V., and Tomatis, L. (2002). Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. *Environ. Health Perspect.* 110, 125-128.

Unkila, M., Pohjanvirta, R., MacDonald, E., Tuomisto, J. T., and Tuomisto, J. (1994). Dose response and time course of alterations in tryptophan metabolism by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the most TCDD-susceptible and the most TCDD-resistant rat strain: relationship with TCDD lethality. *Toxicol. Appl. Pharmacol.* 128, 280-292.

Vainio, S., Heikkila, M., Kispert, A., Chin, N., and McMahon, A. P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature.* 397, 405-409.

Val, P., Lefrancois-Martinez, A. M., Veyssiere, G., and Martinez, A. (2003). SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nucl. Recept.* 1, 8.

Vinson, G. P. (2003). Adrenocortical zonation and ACTH. *Microsc. Res. Tech.* 61, 227-239.

Virtanen, H. E., Rajpert-De Meyts, E., Main, K. M., Skakkebaek, N. E., and Toppari, J. (2005). Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders. *Toxicol. Appl. Pharmacol.* 207, 501-505.

Visser, J. A., McLuskey, A., Verhoef-Post, M., Kramer, P., Grootegoed, J. A., and Themmen, A. P. (1998). Effect of prenatal exposure to diethylstilbestrol on Mullerian duct development in fetal male mice. *Endocrinology.* 139, 4244-4251.

Walsh, L. P., McCormick, C., Martin, C., and Stocco, D. M. (2000). Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environ. Health Perspect.* 108, 769-776.

Walsh, L. P. and Stocco, D. M. (2000). Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. *Biol. Reprod.* 63, 1024-1033.

Warren, D. W., Huhtaniemi, I. T., Tapanainen, J., Dufau, M. L., and Catt, K. J. (1984). Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology.* 114, 470-476.

Welsh, M., Saunders, P. T., Fisken, M., Scott, H. M., Hutchison, G. R., Smith, L. B., and Sharpe, R. M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J. Clin. Invest.* 118, 1479-1490.

Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Beverdam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev. Biol.* 287, 111-124.

Williams, K., McKinnell, C., Saunders, P. T., Walker, M., Fisher, J. S., Turner, K. J., Atanassova, N., and Sharpe, M. (2001). Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. *Hum. Reprod. Update.* 7, 236-247.

Wilson, J. D. (1978). Sexual differentiation. *Annu. Rev. Physiol.* 40, 279-306.

Wilson, V. S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., and Gray, L. E.,Jr. (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol. Lett.* 146, 207-215.

Wolff, M. S., Toniolo, P. G., Lee, E. W., Rivera, M., and Dubin, N. (1993). Blood levels of organochlorine residues and risk of breast cancer. *J. Natl. Cancer Inst.* 85, 648-652.

Wotus, C., Levay-Young, B. K., Rogers, L. M., Gomez-Sanchez, C. E., and Engeland, W. C. (1998). Development of adrenal zonation in fetal rats defined by expression of aldosterone synthase and 11beta-hydroxylase. *Endocrinology.* 139, 4397-4403.

Yamada, T., Kunimatsu, T., Sako, H., Yabushita, S., Sukata, T., Okuno, Y., and Matsuo, M. (2000). Comparative evaluation of a 5-day Hershberger assay utilizing mature male rats and a pubertal male assay for detection of flutamide's antiandrogenic activity. *Toxicol. Sci.* 53, 289-296.

Yamamoto, M., Shirai, M., Tamura, A., Kobayashi, T., Kohara, S., Murakami, M., and Arishima, K. (2005). Effects of maternal exposure to a low dose of diethylstilbestrol on sexual dimorphic nucleus volume and male reproductive system in rat offspring. *J. Toxicol. Sci.* 30, 7-18.

Yao, H. H., Whoriskey, W., and Capel, B. (2002). Desert Hedgehog/Patched 1 signaling specifies fetal Leydig

cell fate in testis organogenesis. *Genes Dev.* 16, 1433-1440.

You, L., Casanova, M., Archibeque-Engle, S., Sar, M., Fan, L. Q., and Heck, H. A. (1998). Impaired male sexual development in perinatal Sprague-Dawley and Long-Evans hooded rats exposed *in utero* and lactationally to *p,p'*-DDE. *Toxicol. Sci.* 45, 162-173.

You, L. and Sar, M. (1998). Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. *Endocrine.* 9, 253-261.

Yu, R. N., Ito, M., Saunders, T. L., Camper, S. A., and Jameson, J. L. (1998). Role of Ahch in gonadal development and gametogenesis. *Nat. Genet.* 20, 353-357.

Zhang, F. P., Pakarainen, T., Zhu, F., Poutanen, M., and Huhtaniemi, I. (2004). Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice. *Endocrinology.* 145, 1453-1463.

Zheng, T., Holford, T. R., Mayne, S. T., Ward, B., Carter, D., Owens, P. H., Dubrow, R., Zahm, S. H., Boyle, P., Archibeque, S., and Tessari, J. (1999). DDE and DDT in breast adipose tissue and risk of female breast cancer. *Am. J. Epidemiol.* 150, 453-458.

Zimmermann, S., Steding, G., Emmen, J. M., Brinkmann, A. O., Nayernia, K., Holstein, A. F., Engel, W., and Adham, I. M. (1999). Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol. Endocrinol.* 13, 681-691.

Zirkin, B. R. and Ewing, L. L. (1987). Leydig cell differentiation during maturation of the rat testis: a stereological study of cell number and ultrastructure. *Anat. Rec.* 219, 157-163.