3.1 Introduction to Endocrine Systems

Endocrine systems of the body play an essential and pervasive role in both the short-and long-term regulation of metabolic processes. Nutritional, behavioral, and reproductive processes are intricately regulated by endocrine systems, as are growth (including bone growth/remodeling), gut, cardiovascular, and kidney function and responses to all forms of stress. Disorders of any of the endocrine systems, involving both overactive and underactive hormone secretion, result inevitably in disease, the effects of which may extend to many different organs and functions and are often debilitating or life-threatening. Viewed from this general perspective, the threat posed from environmental chemicals with endocrine activity (either agonist or antagonistic) is potentially serious. However, the fact that humans and wildlife are exposed to such chemicals does not necessarily mean that clinically manifest disturbance of the relevant endocrine system will result, because much depends on the level and duration of exposure and on the timing of exposure.

3.2 Scope and Terminology

3.2.1 Overview

The endocrine system originally was considered to consist only of glands that secreted hormones into the blood that traveled to distant target tissues, bound to specific cellular receptors, and produced characteristic actions. Currently, our concept of "endocrine" has been broadened by the discovery of other chemical regulators, such as chemicals secreted into the blood by neurons, that are sometimes termed neurohormones. The term "cytocrine" has been applied to numerous local or intercellular chemical regulators, including growth factors. Intercellular cytocrines that travel through the extracellular fluids to other cells in a tissue also are known as paracrine and autocrine regulators, depending on whether they affect other cells or themselves, respectively. The term "intracrine" has been suggested for intracellular regulators such as second messengers and transcription factors. Even before allowing for the increase in complexity of "endocrinology" that has resulted from recent recognition of the many cytocrine/paracrine systems that operate, it had been realized that there were numerous "classical" endocrine systems in the body that regulate processes as diverse as blood pressure, smooth muscle contraction, fluid balance, and bone resorption.

It is beyond the scope of this chapter to describe the entire endocrine system; instead, the focus will be on the three major endocrine axes that affect reproductive development and function. This restriction is based on the observations that many manifestations of endocrine disruption involve the reproductive system, particularly during its vulnerable developmental period. The particular aspects of the endocrine system that are covered include the HPG, the HPT, and the HPA axes. This restriction is arbitrary and should not imply that endocrine disruptors cannot affect other endocrine axes. It is also emphasized that the general principles on which all endocrine (and probably paracrine) axes are first set up and then operate are essentially identical, and hence, most of what is discussed below can be transferred in principle to other endocrine axes that are not described. The emphasis will be on the vertebrate endocrine system, with only minor attention paid to invertebrates. Although there are many parallels between vertebrate and invertebrate endocrine mechanisms, there are some major differences as well. General discussions of invertebrate endocrinology have been reported (Downer and Laufer, 1983; Matsumoto and Ishii, 1997; Cymborowski, 1992; Nijhout, 1994). This chapter consists of two main parts: sections 3.1–3.11 detail the normal functioning of the endocrine system, both in adults and in the developing organism; sections 3.12–3.16 focus on the impact of endocrine disruptors on organ systems and disease processes. The largest of the sections deals with effects on reproductive system development using several well-characterized examples from the experimental literature (e.g., MXC, vinclozolin, ketaconazole,

List of Abbreviations

17α,20β-P 17α,20β-dihydroxy-4-pregnen-3-one
5-HT Serotonin
ACTH Adrenocorticotropin hormone
AGD Anogenital distance
AhR Aryl hydrocarbon receptor
AR Androgen receptor
ARNT AhR nuclear translocator
AFP α-Fetoprotein
BBP Butylbenzyl phthalate
BNF β-Napthoflavone
cAMP Cyclic AMP
CBG Corticotropin-binding globulin
CRH Corticotropin-releasing hormone
CYP Cytochrome P
DBP Di-*n*-butyl phthalate
DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
DEHP Di-ethylhexyl phthalate
DEP Diethyl phthalate
DES Diethylstilbestrol
DHEA Dihydroepiandrosterone
DHP Dihexyl phthalate
DHT Dihydrotestosterone
DMP Dimethyl phthalate
DOTP Dioctyl phthalate
E_2 17β-Estradiol
EDCs Endocrine-disrupting chemicals
ER Estrogen receptor (α and β isoforms)
FSH Follicle-stimulating hormone
Gal_4-HEGO Gal_4-human estrogen receptor construct
GH Growth hormone
GnRH Gonadotropin-releasing hormone
GSI Gonadal-somatic index
GTH Gonadotropin (isoforms I and II)
HIF-1α Hypoxia inducible factor 1α
HPA Hypothalamic-pituitary-adrenal
HPG Hypothalamic-pituitary-gonadal
HPOA Hypothalamic preoptic area
HPT Hypothalamic-pituitary-thyroid
HPTE 2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane
IL Interleukin
IUGR Intrauterine growth retardation
LE Long-Evans
LH Luteinizing hormone
LOAEL Lowest observed adverse effect level
M1, M2 Vinclozolin metabolites
MEHP Mono-ethylhexyl phthalate
MIH Müllerian inhibiting hormone
MIS Anti-Müllerian substance
mRNA Messenger RNA
MXC Methoxychlor
NOAEL No observed adverse effect level
PCBs Polychlorinated biphenyls
PCDFs Polychlorinated dibenzofurans
PGs Prostaglandins
PRL Prolactin
SARMs Selective androgen receptor modulators
SD Sprague-Dawley
SERMs Selective estrogen receptor modulators
SHBG Sex hormone–binding globulin
T_3 Triiodothyronine
T_4 Thyroxine
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TRH Thyrotropin-releasing hormone
TSH Thyroid-stimulating hormone
US EPA United States Environmental Protection Agency

phthalates, and dioxin). These examples were selected to provide a broad view of the basic modes of action that are involved in the interaction of chemicals with the endocrine system. In addition to describing the modes of actions, descriptions of the critical periods, dose sensitivity, and resulting phenotypes seen in experimental models are provided. Similarly to the section on normal endocrine function, this section deals primarily with effects on vertebrates, and mammals in particular. Succeeding sections provide examples of EDC-related modes of action pertinent to carcinogenesis and the function of the nervous and immune systems. The final section provides a overall framework to judge whether a particular outcome, whether observed in the laboratory, in the field, or in an epidemiology setting, could be related to an EDC-related mode of action. This framework is intended to provide a structure by which subsequent observations, either contained in this assessment or reported subsequently in the scientific literature, can be judged relative to ascertainment of the mode of action.

3.2.2 Homeostasis

The fundamental role of all endocrine systems is to enable a dynamic, coordinated response of a distant target tissue to signals originating from another organ and, in some instances, cues originating from outside of the body. For most endocrine systems, the primary objective is to maintain some form of "homeostasis," avoiding wild swings in hormone levels/responses that might otherwise have detrimental metabolic effects (Norman and Litwack, 1998). A good example is the role of insulin in maintaining blood glucose levels within the normal range, that is, a range that does not fall so low as to result in unconsciousness and does not rise so high that wasteful excretion/spillage into urine occurs. When insulin levels do not respond to changing blood levels of glucose, diseases such as diabetes are the result. All endocrine systems operate to a large extent on the "seesaw" principle (Figure 3.1), in which the target cells send feedback signals (usually negative feedback) to the regulating cells, with the result that secretion of the target cell–stimulating hormone is altered (usually reduced) by one or more of the products of the target cells (Darlington and Dallman, 1995). However, in reality, there are usually elaborations or refinements of this simple archetypal endocrine system that enable all of the endocrine systems of the body to be integrated via cross talk. The reasons for this are obvious. For example, reproduction needs to take account of age, nutritional status, and in most animals, season of the year. Similarly, stress responses, and to a lesser extent, endocrine systems regulating hunger, need to be able to override other endocrine systems when danger threatens. This cross talk is vital for a healthy life and has important implications for the evaluation of endocrine disruptors. Exposure to an estrogenic chemical, for example, may affect not only the reproductive endocrine axis but also several other endocrine systems as well as bone, fat, and cardiovascular systems.

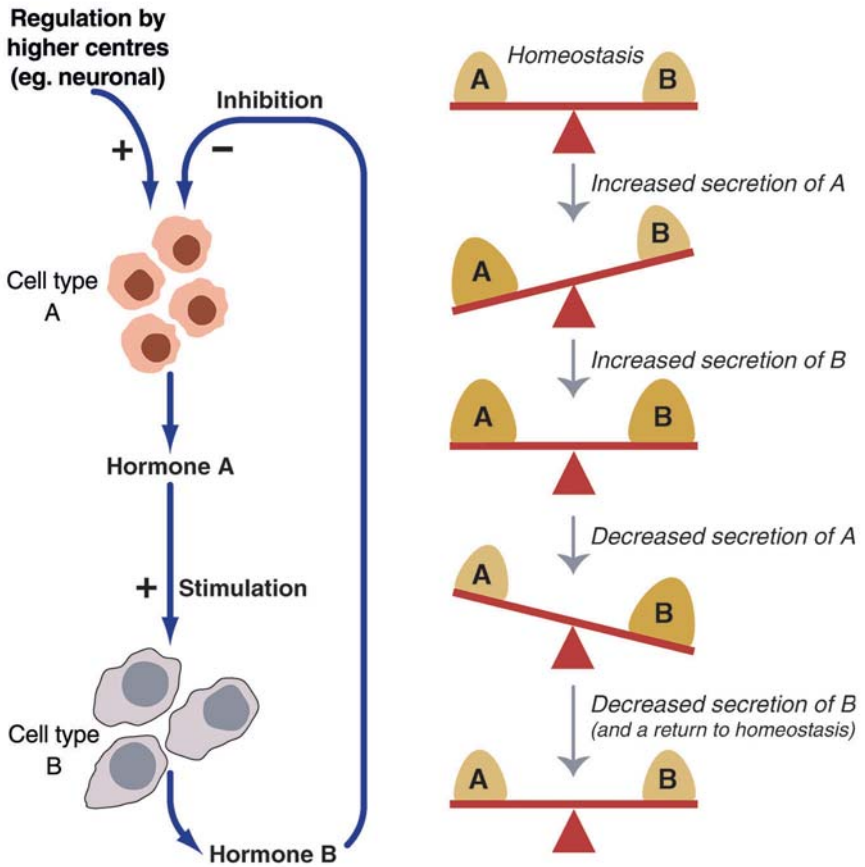

Figure 3.1 - Schematic diagram illustrating the basic "seesaw" principle on which endocrine systems work. Cell type A secretes hormone A, which regulates production of hormone B by cell type B, and in turn, hormone B exerts negative feedback regulation of the secretion of hormone A. In this way, swings in secretion of hormone A or B will be compensated for to maintain homeostasis (i.e., the correct levels of A and B), as shown on the right. This general principle operates in most, if not all, endocrine and paracrine systems, although in reality there are usually additional factors that will interplay in the regulation of levels of A and B.

3.2.3 Programming of Endocrine Axes

Although homeostasis, via seesaw-type mechanisms, is a central feature of all endocrine systems, it should be stressed that the balance between the two sides of the "seesaw" need to be set up or programmed before the system will work correctly. This programming will determine at what level the two sides of the seesaw will begin to respond to signals from the other side (Figure 3.1). For many of the endocrine systems, it appears that the setup program is established during fetal/neonatal development in mammals and that an abnormal environment at this stage of life can result in permanent misprogramming (De Kloet et al., 1988; Seckl, 1999). A good example of this is what happens as a result of fetal IUGR. Although such offspring often reach normal growth postnatally, they show a high incidence of insulin resistance (higher than normal insulin levels) and, consequently, are at increased risk of diabetes, obesity, and cardiovascular disease in later life; they are also prone to precocious puberty. These changes are believed to represent an adaptation of the fetus to its suboptimal nutritional supply and may result from elevation of glucocorticoid levels in the fetus (Philips et al., 1998). A more specific example concerns programming of the hypothalamus of the female, but not of the

male, to respond to gradually rising estrogen levels by triggering a positive response—the ovulatory GnRH-driven LH surge. In mammals, this programming is established perinatally, and exposure of the female at this time to moderate levels of male sex steroids will prevent this programming and render the female permanently infertile because of anovulation (Dohler, 1991). In contrast, exposure of the adult female to the same male sex steroids will not alter this programming, although it may temporarily disrupt ovulation by increasing negative feedback (Figure 3.2).

3.2.4 Impact of Endocrine Disruptors

In considering the potential impact of endocrine disruptors on bodily functions, the following points are critical :

(1) Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effect.
(2) Exposure during the period when programming of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals.
(3) Exposure to the same level of an endocrine signal at different stages in the life history or in different seasons may produce different effects.
(4) Because of cross talk between different endocrine systems, effects may occur unpredictably in endocrine systems other than the system predicted to be affected. This is true for each of the situations in (1) through (3) above.
(5) In view of (4), considerable caution should be exercised in extrapolating *in vitro* measures of hormonal activity to the situation *in vivo*.

3.3 The HPG Axis in Mammals

3.3.1 Overview of the HPG Axis

This axis (Figure 3.2) involves three component parts: 1) GnRH neurons projecting from the hypothalamus of the brain; 2) gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins LH and FSH;, and 3) the somatic cells of the gonads (theca and granulosa cells in the ovary, Leydig and Sertoli cells in the testis). GnRH is secreted in pulses (Kimura and Funabashi, 1998; Terasawa, 1998) from the terminals of GnRH neurons and acts on the gonadotropes to induce secretion of both LH and FSH, which then act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). Secretion of GnRH is modified by other neurons (e.g., Crowley, 1999), and the actions of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides (Evans, 1999). As a consequence, gonadal sex steroids stimulated by LH and the protein hormone inhibin (the A form in females, the B form in males) stimulated by FSH are released into the bloodstream and provide feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH, and FSH, with inhibin selectively inhibiting FSH and the sex steroids inhibiting LH secretion (Crowley et al., 1991). This description implies that the arrangement of stimulatory and negative feedback loops complies with the simple arrangement shown in Figure 3.1. In reality, the arrangement is more complex and sophisticated. For example, the effects of GnRH on LH and FSH secretion are radically different, with LH release being stimulated very acutely (in

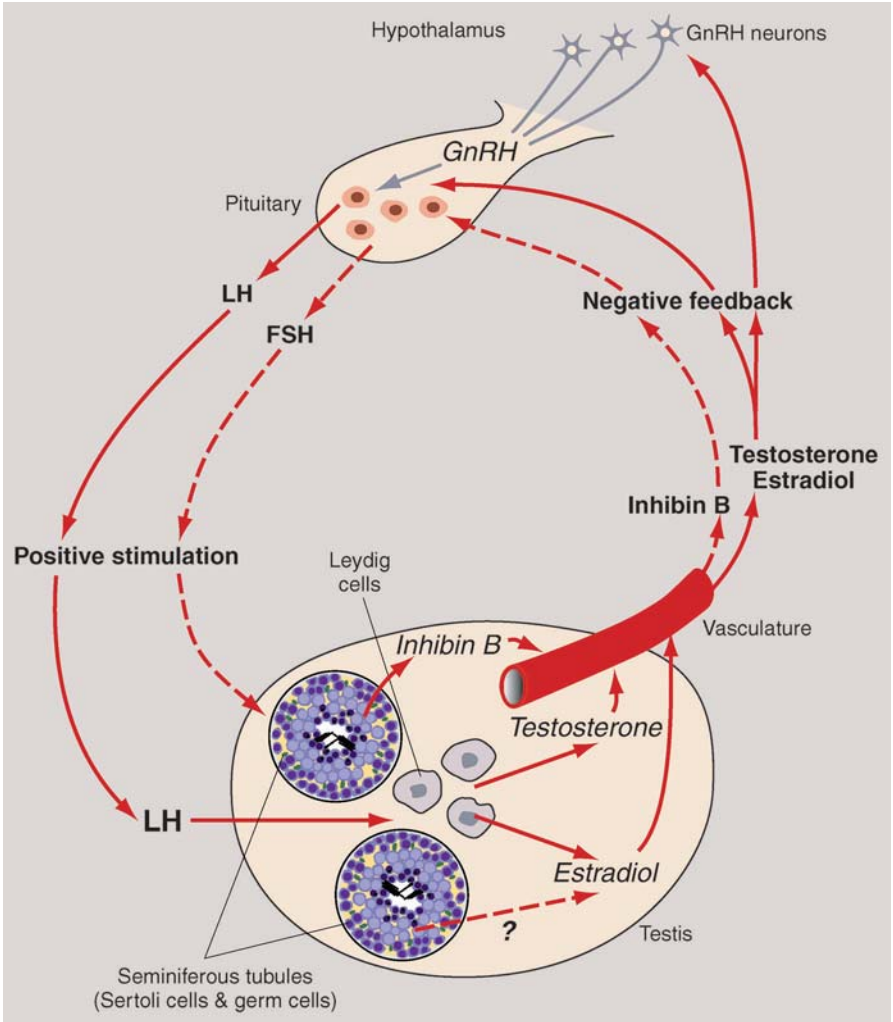

Figure 3.2 - Diagrammatic representation of the main working components of the mammalian HPG axis. The decapeptide GnRH is secreted from the terminals of GnRH neurons into the portal blood system, which delivers this "message" to gonadotrope cells in the anterior pituitary gland that express receptors for GnRH. Binding of GnRH to these receptors stimulates the synthesis and secretion into the bloodstream of the two gonadotropins LH and FSH. The gonadotropins then travel via the systemic bloodstream to reach their distant target cells in the gonad (a testis is shown for example). LH acts on Leydig cells to stimulate synthesis and secretion of testosterone, which in turn gains access to the bloodstream and via effects on the hypothalamus and anterior pituitary gland suppresses synthesis and secretion of GnRH and LH, respectively (= negative feedback). Similarly, FSH acts on Sertoli cells to drive the secretion of a protein hormone, inhibin B, which then travels via the bloodstream to the pituitary gland to suppress the synthesis and secretion of FSH (= negative feedback). Note that some of the negative feedback effects of testosterone may occur via its conversion to E_2, either in the testis (by Leydig cells and/or germ cells) or in the hypothalamus/pituitary gland. Note also that testosterone and/or E_2 exerts effects at many sites other than the hypothalamus and pituitary gland and that paracrine effects of these hormones, especially of testosterone within the testis, are also of vital importance. These and other refinements of the basic system illustrated here are outlined in the text.

pulses) by the GnRH pulses, whereas the response of FSH is extremely sluggish and takes many hours (Crowley et al., 1991; Bousfield et al., 1994). This stems from fundamental differences in GnRH-induced synthesis, packaging, and release of LH and FSH. Similarly, although the sex steroids (primarily testosterone in the male, E_2 in the female) negatively regulate LH secretion via effects on both GnRH secretion and gonadotrope function, they also exert some negative feedback on FSH secretion; in contrast, inhibin selectively inhibits FSH secretion.

3.3.2 Target Cell Sensitivity

In addition to these minor refinements of the archetypal endocrine system, there are other important factors that must be considered. One such factor is modulation of target cell sensitivity to stimulation. Gonadotropes do not exhibit a constant, unchanging response to GnRH stimulation, nor do the target cells in the gonads maintain unaltered responsiveness to LH/FSH stimulation. Sensitivity of the target cell to its stimulator is regulated both acutely and chronically (Conn, 1994; Erickson and Schreiber, 1995). For example, an abnormally high frequency of GnRH pulses or chronic exposure to GnRH or to agonistic analogues of GnRH (which are more resistant to degradation) results in loss or down-regulation of GnRH receptors on the gonadotropes, which serves to make them more resistant (= less sensitive) to further stimulation. This happens in a matter of hours and is followed more slowly by the gradual development of "desensitization" that involves changes to the GnRH-stimulated second messenger signaling mechanisms that reduce overall responsiveness of the gonadotropes to GnRH. Analogous mechanisms operate in gonadal cells to regulate their responsiveness to LH and, to a lesser extent, to FSH. In other words, each target cell in the endocrine axis also regulates its own responsiveness to stimulation. There is still further refinement of this process via cross talk between neighboring cells, especially in the gonads (Leung et al., 1992). There is good evidence, for example, that Sertoli cells in the testis are able to modulate both the numbers of LH receptors expressed in neighboring Leydig cells and their steroidogenic responsiveness via altering expression of steroid synthetic enzymes (Sharpe, 1993). In return, the testosterone secreted by Leydig cells exerts important paracrine regulatory effects on Sertoli cell function (Sharpe, 1994).

3.3.3 Metabolism of Endocrine Hormones

A further potentially modulable element in the component loops of the HPG axis is the metabolism of the secreted hormones. Increased or decreased catabolism, with a consequent change in half-life of a hormone, will change its effectiveness without altering its level of secretion. FSH has a naturally longer half-life than LH (i.e., it is metabolized more slowly), which is one reason why changes in FSH levels are more sluggish than changes in LH (Bousfield et al., 1994). Of much more importance is the role of proteins that bind the sex steroids. These include albumin and AFP in the fetus/neonate and, most important, SHBG in humans. Approximately 97–98% of testosterone and E_2 that circulate in blood in humans is bound to SHBG, and only 2–3% is free and thus biologically active (Moore and Bulbrook, 1988; Rosner, 1990). This arrangement has two important consequences: 1) the half-life of the sex steroids is considerably prolonged and 2) a new indirect pathway for regulating sex steroid action becomes evident: modulation of SHBG secretion (by the liver) can potentially alter levels of bioactive sex steroid without affecting any of the major component parts of the HPG axis. In practice, the main (stimulatory) regulators of SHBG production are the sex steroids themselves as well as other important regulators of SHBG production that are components of other endocrine systems (Moore and Bulbrook, 1988; Rosner, 1990). Similar arguments may apply to other binding proteins (e.g., AFP).

3.3.4 Interaction of Paracrine and Endocrine Components of the HPG Axis

Testosterone produced by Leydig cells acts on neighboring Sertoli cells (an example of a paracrine effect). This is arguably the most important role of testosterone in the male, as its effect on Sertoli cells is the main pathway via which spermatogenesis is supported (Sharpe, 1994). There are analogous effects in the ovary with androgens produced by the theca cells exerting paracrine effects on granulosa cells in the adjacent, developing follicle (Erickson and Schreiber, 1995). The most important consequence of the exposure of granulosa cells to testosterone is that they are then able to convert this androgen to E_2, which then exerts multiple endocrine effects in the uterus and elsewhere in the body, including its role in negative feedback. This conversion of testosterone to E_2 also occurs at many other sites in the body, in both the male and the female (Simpson et al., 1997; Sharpe, 1998). The ability of cells to express aromatase and/or 5α-reductase, and thus to transform an endocrine hormone (testosterone) into a locally acting paracrine hormone (E_2 or DHT; Figure 3.3), appears to be far more common (especially in the male) than was initially hypothesized. These sites of paracrine action obviously depend on the supply of substrate and thus on the main endocrine axis, and "leakage" of the paracrine-generated products into the general circulation may contribute to negative feedback, although conceptually this would not appear to be important. One or both of the component parts of the paracrine mechanisms illustrated in Figure 3.3 are now known to be expressed in bone, muscle, the cardiovascular system, adipose tissue, the pituitary gland, and brain as well as throughout the reproductive systems of both male and female (Simpson et al., 1997; Sharpe, 1998). Paracrine systems can be considered to act as local satellites of the major endocrine axis, their role being to serve local needs.

3.3.5 Developmental Role of the HPG Axis

As noted, the setting up of endocrine axes takes place largely during fetal/neonatal development. During this period, feedback sensitivity of the hypothalamus and pituitary gonadotropes to steroids from the gonads is established, and this will determine at what level of sex steroid a reduction in GnRH and/or LH/FSH secretion will be triggered. The details of how this occurs are incompletely understood but clearly involve programming of neuronal pathways (Dohler, 1991). At the same time, differences between male and female feedback centers are programmed (Dohler, 1991; Gorski, 1996). This is necessary because female reproduction usually operates around a reproductive cycle, for example, the estrous or menstrual cycle, whereas male reproduction is usually a continuous or more protracted, noncyclic event. Production of gonadal hormones in the female is therefore cyclic whereas in the male it is relatively uniform, apart from special periods such as puberty and seasonal infertility. Appropriate changes to the "wiring" of the hypothalamus of the male and female therefore have to be induced during development to ensure that the pituitary gland of an adult female will respond as in a female rather than as in a male. Testosterone produced during fetal/neonatal life plays a role in programming the development of a "male" hypothalamus and brain, and administration of testosterone to a female during this critical programming period will result in masculinization of hypothalamic

function and consequent acyclicity and anovulation in adulthood (Dohler, 1991; Gorski, 1996). The relative roles of testosterone, DHT, and E_2 vary from behavior to behavior in a species-specific manner. This is true for both organizational and activational influences. In some species, all three hormones play a role in masculinization (Cooke et al., 1998). Importantly, there are significant species differences in the organizational and activational control of the development of sexually dimorphic behaviors (Cooke et al., 1999). For example, in the rat, activation of malelike mounting behavior in the adult female rat does not require an organizational effect of hormones during prenatal life. In at least some strains of rats, this behavior can be activated by testosterone in adult females. In contrast to the rat, androgens play an important role in the organization of mounting behavior in the nonhuman primate (Goy, 1978; Pomerantz et al., 1985). This difference must be considered when extrapolating findings from the rat to humans. Rough-and-tumble play behavior is one of the few social behaviors that appears to be regulated by androgens in both the rat and rhesus monkey (Goy, 1978).

3.3.6 Role of Hormones in Mammalian Sex Differentiation

As well as leading to masculinization of the brain, perinatal testosterone secretion by the testis is also responsible for masculinization of the body in general. This involves the formation of male genitalia, but effects on the many organs throughout the body also occur at this time (Simpson and Rebar, 1995). The role of androgens in sex differentiation is well understood. Before sex differentiation, the mammalian embryo has the potential to develop a male or a female phenotype. Following gonadal sex differentiations, testicular sections induce differentiation of the male duct system and external genitalia. The development of phenotypic sex includes persistence of either the Wolffian (male) or Müllerian (female) duct system and differentiation of the external genitalia. Generally, masculinization of the tissue/organ in question results from local conversion of circulating testosterone to either DHT or E_2, as shown in Figure 3.3, but for some tissues, MIS is also involved.

The female avoids developing as a male by simply not switching on secretion of testosterone by the ovary, and it is largely the absence of this endocrine signal that results in phenotypic and endocrinotypic female development (Simpson and Rebar, 1995). The central role of testosterone in facilitating masculinization has two important downsides. First, if a genotypic male fails to make testosterone, it will not masculinize and will develop as a phenotypic female (but with testes). Second, and conversely, if a genotypic female is exposed to sufficient testosterone (or other androgens), she will be masculinized (but will have ovaries). There are numerous examples of both of these situations that result most commonly from inactivating mutations (e.g., in the AR, resulting in lack of masculinization in males) or from abnormal androgen production (usually by the adrenal glands) by the mother or female fetus that lead to masculinization of the developing female (Simpson and Rebar, 1995). It is also emphasized that these are not necessarily all-or-nothing events. Partial masculinization of the female or partial failure of masculinization of the male can occur, including potentially quite subtle effects. Where these affect the genitalia or other external phenotypic feature (e.g., presence/absence of horns), they may be easily detected, but if the effects are confined to the brain or to another organ, they may not be easily deducible.

Like the brain and the genitalia, other organ systems such as the liver and muscles are also "imprinted" by the hormonal milieu during development and hence may also be targets of xenobiotics that perturb the normal endocrine profile at various stages of life. For example, the development of the levator ani-bulbocavernosus muscles and their neural regulation has been employed as a model of organizational and activational roles of testosterone on the ontogeny of sexual dimorphisms in the rat (Breedlove et al., 1999). These tissues are also sexually dimorphic in humans, an effect whose critical period lies in the first trimester of pregnancy. The levator ani muscles and spinal nuclei of the bulbocavernosus are considerably larger in males than in female rats, a response that requires testosterone exposure during both the prenatal and postpubertal stages of life. As the levator ani lacks 5α-reductase, testosterone, and not DHT, is the hormone that initiates the malelike developmental pattern.

Perhaps the most important aspect of these various "programming" changes is their irreversibility. The greatest concern about environmental hormone disruptors is centered on the

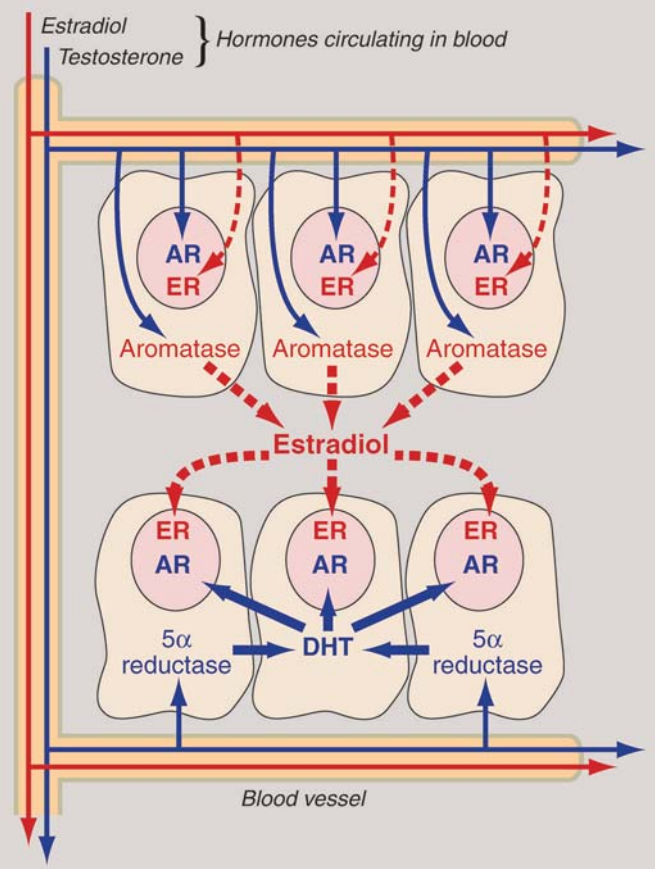

Figure 3.3 - Schematic illustration of the interplay between endocrine and paracrine regulatory systems as exemplified by androgens and estrogens. Testosterone and E_2 both circulate in the bloodstream as endocrine hormones, but within specific cell types testosterone can be converted to E_2 (via the enzyme aromatase), which can then interact with ERs or be converted to the more potent androgen, DHT, which can interact with ARs. Both of these conversion steps effectively amplify a relatively weak "endocrine" signal into a more powerful "paracrine" signal that can act locally on target cells. Because E_2 deriving from systemic and local sources will interact with the same ERs, and similarly, both testosterone from the systemic bloodstream and DHT derived locally will interact with the same ARs, this illustrates how endocrine and paracrine systems can interplay in a potentially powerful way using very simple mechanisms. The generation of locally high hormone levels to exert paracrine effects represents a means of regulating target cell function according to local needs of the tissue in question while still maintaining an overall endocrine influence on the same tissue via the blood-derived hormones.

possibility that exposure of an animal to such an agent during perinatal life can result in a permanent adverse or abnormal change; exposure to the hormone disruptor does not need to be chronic, as transient exposure at a critical time during development is all that is required. There is added uncertainty in this regard because of emerging understanding about how different androgens and estrogens may act in different tissues—so-called SERMs (Cosman and Lindsay, 1999) and corresponding SARMs (Negro-Vilar, 1999). The ability of such compounds to selectively activate or antagonize estrogen or androgen pathways in specific tissues presages the discovery of similar activities for certain environmental chemicals. Predicting the effects of such compounds in the context of programming of development of the reproductive system and its endocrine axis is extremely difficult.

3.3.7 The HPG Axis in Nonmammalian Species

Nonmammalian vertebrates differ greatly from mammals and one another in their modes of reproduction, with patterns of sequential and simultaneous hermaphroditism, parthenogenesis, viviparity, and gonochorism found in many major groups (van Tienhoven, 1983). Additionally, they may be more limited in their breeding frequencies. Some species breed only once (semelparous), whereas others may breed two or more times (iteroparous). The time of gonadal activity may be very short, with the gonads remaining quiescent during most of the year. Dissociated reproduction where testicular and ovarian development actually occurs at different times of the year also is known for numerous species (e.g., Houck and Woodley, 1994). However, the HPG axes of these animals are surprisingly similar in their operation, in the pattern of feedback mechanisms, and in the hormones involved to that described for mammals (for reviews, see Norris, 1997; Bentley, 1998).

GTH release is controlled in all nonmammals by a GnRH decapeptide molecule similar to that in mammals (Sherwood et al., 1994; Sower, 1998). As in mammals, these cells develop in the nasal placodes and migrate into the preoptic area and hypothalamus during early development (Dellovade et al., 1998). Typically, at least two forms of GnRH are found, but the second form (usually chicken GnRH-II, first isolated from chickens) functions primarily as a neurotransmitter or neuromodulator and probably influences reproductive behaviors rather than the HPG axis. Furthermore, many teleosts have three forms of GnRH present in the brain. A major difference occurs in the delivery of GnRH in teleostean fishes that lack a hypothalamo-hypophysial portal system between the hypothalamus and the pituitary and exhibit direct penetration of the adenohypophysis by GnRH axons. A portal delivery system also is lacking in the jawless fishes (agnathans), in which diffusion is the mode of delivery (Gorbman et al., 1999).

There are two distinct GTHs that are not directly homologous to mammalian FSH and LH. The first GTH, called GTH-I, is responsible for gonadal growth and gamete formation. The second, GTH-II, is involved with gamete release. Administration of sufficient amounts of either GTH can produce the effects of both, but they are secreted sequentially *in vivo*. Among the tetrapods (amphibians, reptiles, birds, and mammals), only the squamate reptiles (lizards, snakes) appear to have a single, FSH-like GTH, whereas all others produce both FSH-like and LH-like GTHs.

In general, testosterone is the major androgen produced in all vertebrates, and E_2 is the major estrogen. Many male teleosts also produce 11-ketotestosterone, and it is the predominant circulating androgen in many species. Female teleosts also produce testosterone, and circulating levels of testosterone may be as great as for E_2. Teleosts also produce important progesterone-like molecules, 17α,20β-P and 17,20β,21 trihydroxy-4-pregnen-3-one that cause final oocyte maturation and ovulation. It is secreted under the influence of GTH-II. This steroid may have pheromonal roles in mating as well. In some teleosts, the corticosteroid deoxycorticosterone produced by adrenocortical cells has been shown to induce final oocyte maturation and ovulation. Tetrapods secrete testosterone, E_2, and progesterone, which all play reproductive roles similar to those observed in mammalian development and reproduction. A secondary androgen secreted by all tetrapods is DHT. Female amphibians, like teleosts, exhibit high levels of androgens as well as estrogens during the reproductive portions of their life histories, but normally androgens are not prominent in the blood of female reptiles and birds. Knowledge of the mechanisms for steroid actions on target cells and characteristics of steroid appear to be similar to mammalian systems, although there are several differences. For example, in addition to ER-α and ER-β, a third distinct subtype, ER-γ, has been identified in teleosts (Hawkins et al., 2000). In addition, the teleost progestin receptor differs from its mammalian counterpart in its binding affinity for steroids and does not bind many EDCs that bind to the mammalian progesterone receptor (Pinter and Thomas, 1997). Thus, care must be taken in extrapolating the effects of EDCs across vertebrate taxa.

Complete or partial sex reversals of the gonads can be caused by early exposure of eggs, larvae, or juvenile animals to estrogens or androgens (Burns, 1961; Hayes, 1998a, 1998b), including many paradoxical effects such as feminization by androgens. Additionally, androgens usually inhibit female duct (Müllerian) development while enhancing male duct (Wolffian) development, whereas estrogens do the reverse. Androgens may enhance degeneration of the Müllerian ducts brought about by MIH or MIS. Estrogens are thought to protect oviducts from MIH (Norris, 1997), and paradoxical actions of androgens have also been reported (Norris et al., 1997a, 1997b; Clark et al., 1998). Clearly, endocrine disruptors that mimic estrogens or have antiandrogenic activity could potentially have drastic effects on wildlife exposed during development or as juveniles.

Estrogens can stimulate synthesis of ovalbumin protein by cells of the avian oviduct (Schlinger and Saldanha, 1998) and also the synthesis of vitellogenins by the liver (LeFleur, 1998; Meyer, 1999). Vitellogenins are precursors used by the ovaries to synthesize yolk proteins that are incorporated into eggs. The synthesis of vitellogenin (vitellogenesis) is a dramatic biomarker for estrogenic action in adult vertebrates that produce yolky eggs (fishes, amphibians, reptiles, and birds). Estrogens produce liver hyperplasia and hypertrophy as well as elevation of plasma vitellogenins. If males or immature females are exposed to estrogens, their livers also can be induced to produce vitellogenins (Matthiessen, 1998; Mattthiessen and Sumpter, 1998; LeFleur, 1998; Meyer, 1999). Thus, plasma vitellogenin can be used as a biomarker for exposure to environmental estrogens. In teleost fishes, amphibians, and birds, vitellogenesis is enhanced by PRL or GH, whereas in elasmobranchs, lizards, and turtles, PRL seems to play an inhibitory role (Grau and Weber, 1998). PRL also stimulates parental behaviors and may enhance estrogen-dependent secondary sex characters such as the avian brood patch (Jones, 1971).

Progesterone and progesteronelike hormones regulate non-genomic-based oocyte maturation in amphibians and teleost fishes, respectively (Paolucci et al., 1998), by binding to specific receptors on the cell surface. Oviductal secretion (Chester Jones et al., 1987) and the sensitivity of the amphibian oviduct to contract in the presence of arginine vasotocin (Guillette et al., 1985) are dependent

on progesterone. Progesterone apparently slows development of the young in the viviparous frog *Nectophrynoides ocidentalis* (Bentley, 1998). In turtles, progesterone decreases contractility as it does in mammals (Paolucci et al., 1998). The fact that steroids can induce rapid, cell-surface-mediated nongenomic actions by binding to specific receptors on the cell membrane has implications for the physiological processes they help regulate as well as their disruption by environmental chemicals (Revelli et al., 1998).

3.4 The HPA Axis

3.4.1 Overview of the HPA Axis

This axis operates in a similar way to that illustrated for the HPG axis, the major differences being in the regulatory and secretory molecules involved (Becker, 1995). CRH is secreted from the terminals of hypothalamic neurons and acts on corticotropes in the anterior pituitary gland to regulate the synthesis and secretion of ACTH, which is then transported via the bloodstream to the adrenal glands, where it stimulates the secretion of glucocorticoid hormones (cortisol and/or corticosterone). The latter have numerous effects throughout the body, including important roles in metabolism of carbohydrate, protein and fat, anti-inflammatory effects, and modulation of stress responses (Becker, 1995). As in other endocrine axes, the products of the target cell, the glucocorticoids, exert negative feedback effects at the hypothalamic and pituitary level to suppress CRH secretion. Similar to the sex steroid hormones, much of the glucocorticoid in circulation in blood is bound to a binding protein in the human (CBG), and local release of bioactive hormone from the CBG represents one mechanism of local tissue response to pro-inflammatory changes (Rosner, 1990). Increasingly, it is recognized that glucocorticoids have important "programming" effects during development and that alterations in the circulating levels of these hormones can affect the timing and set points of other endocrine axes. For example, the multiple consequences of IUGR, including the short- and long-term consequences in terms of disease risk, are believed to be triggered largely by elevation of glucocorticoid levels in the fetus (Philips et al., 1998).

Exposure to stresses or elevated glucocorticoid levels may have profound effects on brain development, as revealed by learning and memory deficits in adults. Early effects of handling newborn rats results in better regulatory control of the stress response as well as reduced cell losses in the hippocampus and less memory loss with aging (Francis et al., 1996; Meany et al., 1988). In contrast, elevated glucocorticoids in neonatal rats result in underdeveloped axonal growth as well as a reduction in myelination, formation of dendritic spines, and synaptogenesis, resulting in learning deficits and altered motor function (de Kloet et al., 1988).

There are a number of additional refinements/complexities to the HPA axis outlined above for mammals. Initially, the adrenal glands are a source of several other important hormones, including mineralocorticoids (which act on the kidney), opioid peptides, and enkephalins, as well as catecholamines, all of which have multiple effects throughout the body. Each of these secretions is regulated by mechanisms that are not related to the HPA axis. In the context of reproduction, the most important other products of the adrenal glands are the weak androgens DHEA, DHEA sulfate, and androstenedione, the secretion of which is also stimulated by ACTH. These adrenal androgens may be converted in target tissues to more potent androgens or to estrogens (Figure 3.3) and can therefore potentially affect functioning of the reproductive endocrine axis and the cell types that are responsive to androgens and estrogens (Simpson and Rebar, 1995). Overproduction of adrenal androgens can have major consequences, including partial sex reversal of the female fetus when this occurs *in utero* or in the pregnant mother (above). In the human, adrenal androgens also play a role in early puberty (adrenarche) and are responsible for stimulation of pubic and axillary hair growth (Ritzen, 1998). At the hypothalamic-pituitary level, additional control of ACTH release may be exercised via arginine vasopressin.

3.4.2 The HPA Axis in Nonmammals

Nonmammalian steroidogenic tissue homologous to the mammalian adrenal cortex may be termed the interrenal gland, interrenal tissue, or simply interrenal (Vinson et al., 1993; Norris, 1997a; Bentley, 1998). Hence, the HPA axis often is termed the hypothalamic-pituitary-interrenal axis, especially in fishes. This steroidogenic tissue is often referred to as "adrenocortical" to emphasize its homology to the adrenal cortex of mammals.

The HPA axis of nonmammals, like that of mammals, is stimulated by an initial secretion of a hypothalamic CRH-like peptide, followed in turn by release of ACTH from the pituitary and then secretion of cortisol (most fishes) or corticosterone (most amphibians, birds, and reptiles) from the adrenocortical tissue. The HPA axis is important in regulating responses to stress (Iwama et al., 1997) and appears to have anti-immune actions (Schreck, 1996) similar to those described in mammals (Gaillard, 1994). The organism may adapt to the presence of the stressor with the hormones returning to normal levels. Prolonged stress may be associated with an increase in the activity of the HPA axis, sometimes producing chronically elevated cortisol or corticosterone. In extreme cases, this may lead to exhaustion of the HPA axis and death. Chronically stressed animals may exhibit an activated HPA axis but present with normal or only slightly elevated level of glucocorticoids.

Although many nonmammals have been shown to secrete a mammalian-like CRH, other CRH-like peptides have been discovered in fishes and mammals that can increase ACTH secretion as well. Mammalian ACTH seems to be effective at stimulating adrenocortical secretion in all vertebrates, reflecting the conservation of amino acid sequence among the vertebrates in these peptides we call ACTH. A unique corticosteroid, 1-hydroxycorticosterone, is found among the elasmobranch fishes (sharks, skates, and rays), although they also produce corticosterone.

In teleostean fishes, cortisol functions both as a mineralocorticoid controlling Na^+ and K^+ balance and as a glucocorticoid. Small amounts of corticosterone also have been described in the plasma of teleosts. Larval and aquatic amphibians, like teleosts, produce cortisol as their major corticosteroid, but terrestrial and semiterrestrial amphibians secrete corticosterone, as do all reptiles and birds. Although all tetrapods produce aldosterone, its role in salt balance is not well studied in nonmammals. Mammals have two generalized corticosteroid receptors located in different target cells, respectively, for glucocorticoids and mineralocorticoids. The number of receptor types for corticosteroids in other vertebrates has not been examined intensively.

3.5 The HPT Axis

3.5.1 Overview of the HPT Axis

This axis operates in a very similar way to that illustrated for the HPG axis. TRH is secreted from the terminals of hypothalamic neurons and acts on thyrotropes in the anterior pituitary gland to

regulate the synthesis and secretion of TSH in mammals (Reed and Pangaro, 1995). TSH is then transported via the bloodstream to the thyroid gland, where it acts to stimulate the synthesis of T_3 and T_4, which are released into the bloodstream and act throughout the body to stimulate general metabolic activity. In practice, the main thyroid hormone released is T_4, although T_3 is biologically much more potent (Reed and Pangaro, 1995). In many target tissues, T_4 is metabolized to T_3, which then exerts its effects, another example of how a simple refinement to an endocrine axis can enable greater modulation and control according to local needs. Most of the circulating T_3 results from metabolic conversion of T_4 by a liver deiodinase. Circulating T_3/T_4 feeds back to the hypothalamus and anterior pituitary gland to negatively regulate TRH and TSH release, thus completing the classical endocrine negative feedback loop (Reed and Pangaro, 1995). Most of the feedback at the level of the pituitary gland is due to T_4 that is converted to T_3 in the thyrotrope. At the top end of this circuit, there is an additional controlling factor, somatostatin, which is released from hypothalamic neurons and exerts negative control of TSH release from the anterior pituitary gland. Somatostatin also plays a key role in the (negative) regulation of GH secretion from the anterior pituitary gland, an endocrine axis that is not discussed in this chapter. However, GH stimulates cell growth whereas TSH (via T_3/T_4) stimulates cell metabolism, showing that control of these two endocrine axes is interlinked at this level.

In the present context, interest in the thyroid endocrine axis stems from a) the demonstration that certain PCBs have antithyroidal activity, that is, can antagonize the effects of T_3/T_4 levels (Gray et al., 1993; Porterfield and Hendry, 1998); and b) the important role that the thyroid axis plays in terminal differentiation of various tissues, extending from neurons to muscle and to Sertoli cells in the testis. Many of the actions of thyroid hormones are permissive in that they affect the capacity of cells to respond to other stimuli. For example, the levels of the important enzyme adenyl cyclase, responsible for generation of the second-messenger cAMP in target cells for GH, is enhanced by thyroid hormones.

3.5.2 The HPT Axis in Nonmammals

The structure and functions of the HPT axis of nonmammals are very similar to those of mammals (McNabb, 1993; Norris, 1997b). The thyroid gland has a follicular arrangement, and the mechanisms of thyroid hormone synthesis and secretion as well as peripheral deiodination of T_4 to T_3 are very similar. Patterns of metabolic degradation and excretion also are similar. One major difference in bony fishes is the diffuse nature of the thyroid follicles that are not encapsulated by a connective tissue covering, such that individual follicles are distributed among the connective tissue elements between the second and fourth aortic arches. In some cases, thyroid follicles spread to other organs, including the kidney, liver, and gonads; hence, surgical thyroidectomy is not feasible in these animals.

The major differences in hypothalamic and pituitary regulation reside at the hypothalamic level. TRH is not the major stimulator of TSH release in fishes and amphibians. It appears that CRH is the principal releaser of TSH in amphibians (Denver, 1997). However, mammalian TSH is effective at stimulating iodide accumulation by the thyroid gland and secretion of thyroid hormones in all vertebrates. Thyroid hormones play critical roles in embryonic and postembryonic development of all vertebrates, especially as related to the nervous system. They are also important for the metamorphosis of larval fishes and amphibians into the juvenile body form (Dickhoff et al., 1990; Galton, 1992; Kikuyama et al., 1993; Shi, 1994). These actions not only bring about dramatic morphological changes but also involve biochemical adaptations related to marked changes in habitat and diets, that is, the roles of thyroid hormones and their interactions with PRL and corticosteroids in the smoltification of salmonid fishes and in amphibian metamorphosis. The parr-to-smolt transformation (smoltification) in juvenile salmonids fishes occurs prior to their seaward migration. Studies of coho salmon smoltification (Dickhoff et al., 1990) have documented two- to sixfold transient increases in T_4 and cortisol, respectively, during this time as well as early surges in insulin and PRL. GH also increases and levels remain elevated in smolts.

In larval amphibians, metamorphosis to the juvenile body form involves transient increases in thyroid hormones and corticosteroids as well as a late, brief rise in PRL. In addition, specific changes in the types of deiodinase enzymes result in decreasing conversion of T_4 to the inactive metabolite reverse T_3 and increases conversion of T_4 to the more active T_3 (Galton, 1992). Additionally, changes in thyroid hormone receptor types also occur during metamorphosis (Wolffe et al., 2000). Numerous gene products are up-regulated by thyroid hormones in responding tissues, and a few are down-regulated during metamorphosis (Shi, 1994).

Shedding of skin in salamanders and in reptiles is controlled by thyroid hormones. In contrast, thyroid hormones augment feather loss in birds, which is stimulated by gonadal steroids. These effects of thyroid hormones are similar to their effects on hair replacement in mammals (Norris, 1999).

Thyroid hormones also work synergistically with GH to provide maximal growth rates in fishes, adult amphibians, birds, and possibly reptiles, although the latter have been less studied (Norris, 1997). Finally, thyroid hormones are important stimulators of sexual maturation and are essential for seasonal reproductive events in a wide variety of animals (Norris, 1999). The roles of thyroid hormones in controlling metabolic rates, body temperature, and thermogenesis evolved independently in accordance with homeothermy in both mammals and birds, and these hormones do not play similar roles in fishes, amphibians, and reptiles (Oppenheimer et al., 1995).

3.6 The Pineal Gland: A Photoperiodic Transducer

In mammals, the pineal gland is located above the thalamus of the brain between the cerebral cortices. Through its nocturnal secretion of the biogenic amine melatonin, the pineal has effects on the regulation of many internal physiological rhythms and may provide an important clue for translating photoperiodic stimuli into action. Furthermore, melatonin can alter coat pigmentation and hair growth; can inhibit hypothalamic regulation of the HPA, HPT, and HPG axes; and has been shown to enhance the immune response system (Norris, 1999). Photic input in birds and mammals is accomplished primarily via the optic visual system. However, the pineal of most fishes, amphibians, and reptiles also plays an important role as a direct photoreceptor, and through secretion of melatonin, it may be an important modulator of the HPA, HPT, and HPG axes as well. Any environmental factor that alters pineal function may have profound effects on the well-being of vertebrates.

3.7 Interactions of the HPG Axis with Other Endocrine Systems

The various endocrine axes of the body do not function as isolated islands, as this would clearly compromise the ability of an organism to react and adapt to changing circumstances, for example, season, food supply, and presence of predators. Various components of

other endocrine axes are able to exert important modulatory effects on the HPG axis to alter the timing or efficiency of reproduction. The complexity of such interactions is illustrated in Figure 3.4, which highlights some of the overlapping cross talk. To give further insight into the function and complexity of these interactions, four additional points (with examples) are emphasized.

3.8 Growth in Understanding of Endocrine Systems

New pathways of communication and functional overlap between the various endocrine systems are still being discovered. One example is the relatively recent discovery of leptin following studies of the genetically obese (*ob/ob*) mouse (Rosenbaum and Leibel, 1998). This hormone is produced by adipocytes and exerts important effects on feelings of satiety and hunger and on the appropriate behaviors such as feeding. As fat cell energy reserves are an important component of the insulin–glucose endocrine system, it is known that insulin can exert both direct and indirect (i.e., by altering fat deposition) effects on leptin levels (Figure 3.4). There are also important interactions between leptin and the reproductive system (Friedman and Halaas, 1998; Rosenbaum and Leibel, 1998). An animal reproduces only when the mother has appropriate energy reserves and food supply is good. Leptin provides the necessary signal that unites these various components. When food supply and maternal energy (= fat) reserves are low, elevated leptin can suppress function of the reproductive system. Such pathways play an important role in the timing/initiation of puberty, in regulation of seasonal reproduction, and in certain diseases, for example, the cessation of normal menstrual cycles in women with eating disorders such as anorexia. Although the existence and implications for leptin in nonmammalian vertebrates remain to be explored, the importance of nutritional state to reproduction is known to be as critical as the relationship described in mammals and may exist in all vertebrates.

Another recently recognized complexity of the endocrine system involves the capability of steroids to exert rapid, nongenomic actions by binding to receptors on the cell surface of target cells and activating signal transduction pathways leading to biological responses, in addition to effects mediated by the classic mechanisms of binding to nuclear steroid receptors leading to changes in gene expression (Watson and Gametch, 1999). Specific membrane receptors and rapid nongenomic actions for estrogens and androgens have recently been identified throughout the reproductive system in vertebrates, including the hypothalamus, pituitary, gonads, gametes, steroidogenic cells, and primary and secondary reproductive structures such as the breast (Revelli et al., 1998). Nongenomic steroid actions have been shown to have important functions in several reproductive processes, such as the activation of sperm in mammals (Luconi et al., 2001), electrolyte and fluid transport in the efferent duct of the testes (Leung et al., 2001), and oocyte maturation in fish and amphibians by progestins (Thomas et al., 1998), as well as nonreproductive processes such as chondrocyte proliferation, differentiation, and matrix formation, but their physiological role in most tissues remains unclear. This area is likely to see increased attention in the near future and further complicate the detection and characterization of the full range of effects elicited by EDCs.

3.9 Developmental/Programming Effects of Endocrine Systems

Cross talk between the endocrine systems may have different consequences at different stages of life. Of particular importance are the radical effects that may result from changes to an endocrine axis during the phase when it is "being set up," that is, when thresholds for stimulatory and feedback loops are being programmed. Two such effects can result in changes to the reproductive endocrine axis. The first of these is relatively straightforward and involves the thyroid axis. The circulating level of thyroid hormones (T_3/T_4) can affect terminal differentiation of various tissues (e.g., neurons, muscle cells), and recent studies show that this extends to Sertoli cells in the male. Conversion of the Sertoli cell from an immature, proliferative cell to a mature, nonproliferating cell ready to support spermatogenesis is triggered by thyroid hormone levels in the prepubertal period. Subnormal levels of T_3/T_4 (hypothyroidism) result in prolongation of the Sertoli cell proliferative phase, whereas conversely, supranormal levels of T_3/T_4 (hyperthyroidism) attenuate the Sertoli cell proliferative phase (Sharpe, 1994; Jannini et al., 1995). The net result of such changes is to alter (up or down, respectively) final Sertoli cell number and thereby to alter final testis size and the number of sperm produced per day, because each Sertoli cell can support only a finite number of germ cells. Hypo- and hyperthyroidism have many other consequences in terms of altered body growth and brain development (Figure 3.4), emphasizing again the pleiotropic effects that result from altered function of any individual endocrine axis.

In addition to cross talk, thyroid hormones have important direct effects on differentiation of the brain as well as on its level of activity in adult animals (Akaike et al., 1991; Porterfield and Hendry, 1998). In humans, thyroid deficiencies during gestation or immediately postnatally can produce irreversible mental retardation.

Another, more subtle example of the dramatic consequences of "programming" cross talk between endocrine axes is that of IUGR, which has already been referred to in brief. Small-for-gestational-age offspring are "insulin resistant" as a result of their growth restriction *in utero* and have dramatically lower fat reserves and leptin levels at birth. Such offspring usually exhibit catch-up growth (Jaquet et al., 1999), presumably because of their adapted endocrine changes but remain permanently hyperinsulinemic and insulin resistant. In humans, such individuals are at increased risk of developing diabetes and becoming obese (Philips et al., 1998). Such individuals are also hypertensive and are thus at increased risk of cardiovascular disease, strokes, and kidney disease unrelated to any obesity. The reproductive system is also affected in an adverse way. In the human, IUGR male offspring are at increased risk of cryptorchidism and hypospadias at birth and of developing testicular germ cell cancer and having low sperm counts in adulthood (Sharpe, 1999). Exactly how these changes are induced is unclear but may involve alterations in sex steroid levels. One possible pathway is illustrated in Figure 3.5. It is now well established that hyperinsulinemia results in considerably reduced secretion of SHBG by the liver and a consequent rise in the circulating levels of free (biologically active) sex steroids (Nestler, 1993), and because androgens bind more strongly to SHBG than do estrogens, the androgen/estrogen ratio will be altered. This is dramatically evident in one common disorder of women, polycystic ovarian disease. Such individuals are hyperinsulinemic and have supranormal blood androgen levels that lead to hirsutism as well as polycystic ovaries and anovulatory infertility (Dunaif, 1997). A common risk factor for developing this condition is IUGR.

3.10 Nonreproductive Effects of Sex Steroids

Changes in SHBG resulting from hyperinsulinemia lead to altered androgen and estrogen bioactivity, which, predictably, can alter function of the reproductive system. However, the sex steroids (in

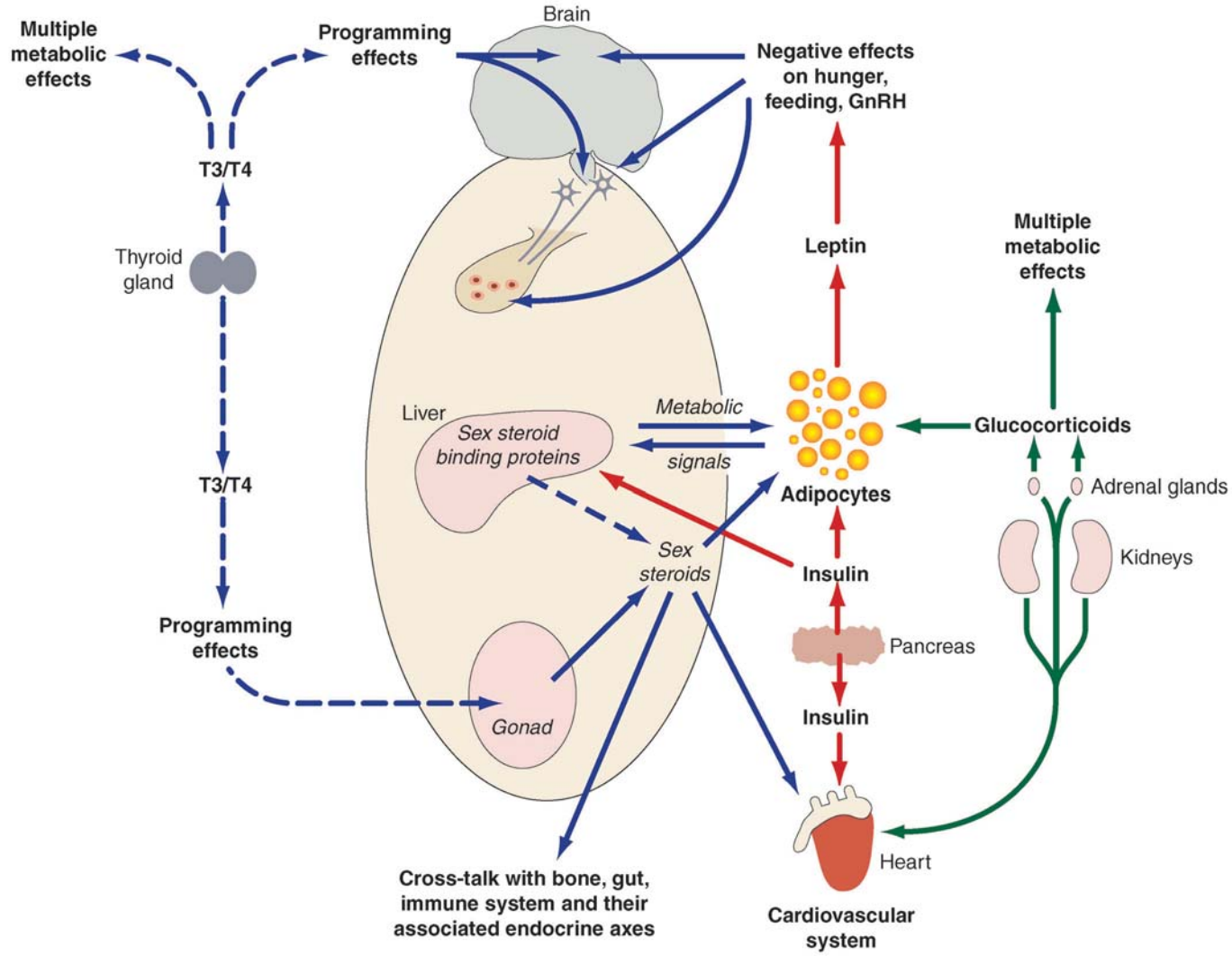

Figure 3.4 - Schematic diagram to illustrate some of the cross talk (integration), which occurs between the mammalian HPG axis (center, shaded) and some of the other endocrine axes of the body. Note that only selective examples are shown and that, in reality, each endocrine axis interacts at multiple levels with other endocrine axes in order to integrate all bodily functions. An important consequence of this complex cross talk is that changes induced in one endocrine axis are likely to lead to changes in other endocrine axes and that such effects can be difficult to predict because of our imperfect understanding of these interactions.

particular, estrogens) also exert multiple other effects throughout the body. Estrogens (and, to a lesser extent, androgens) play a key role in bone formation/resorption in males and females, and estrogen action is essential for epiphyseal closure (Sharpe, 1998). Disorders of sex steroid production or action can lead to osteoporosis or to premature/delayed epiphyseal closure, with consequent effects on final height/body length. Additionally, the sex steroids exert pervasive effects on the cardiovascular system and, in the human, are clearly implicated in gender- and age-dependent changes in risk of developing cardiovascular disease (Sharpe 1998). Multiple effects of the sex steroids on the brain (Gorski, 1996; Meewen and Alves, 1999), digestive system (Sharpe, 1998), immune system (Olsen and Kovacs, 1996), and adipose tissue (Simpson et al., 1997) also occur and, in so doing, will interact with or modulate other endocrine axes that target these tissues (Figure 3.4). Direct or indirect effects on the HPG axis that result in changes in the absolute or relative levels of androgens and estrogens can have pervasive consequences. These effects can be acute/transient (e.g., pituitary feedback effects) or chronic (e.g., bone and cardiovascular effects) in the adult, whereas in the fetus/neonate, any effects that occur may be permanent (e.g., sexual differentiation).

3.11 Endocrine Cross Talk and Endocrine Disruptors

It is emphasized that the prediction of the reproductive consequences of a given chemical from its known sex steroid hormone activity or inactivity is far from straightforward. For example, a dietary change that affects insulin levels (e.g., ingestion of a diet rich in refined sugars) has the potential to alter sex steroid hormone bioactivity via altered production of SHBG (Figure 3.5), but it would not be claimed that refined sugars have sex steroid hormone activity. Even when an environmental chemical is shown to have (weak) steroid hormone activity, it can possess other relevant activities. Thus, the thyroidal activity of PCBs may be as important or even more important than the weak estrogenic/antiestrogenic effects of these compounds when considering their potential impact on the reproductive system. Other environmental chemicals may have antiandrogenic as well as estrogenic effects (e.g., DDT isomers, certain phthalates), which may confuse interpretation of potency *in vivo*. For example, administration of antiandrogens is likely to elevate endogenous estrogen levels. This occurs because antiandrogens block negative feedback loops, which leads to

compensatory elevation of LH levels (Figures 3.1 and 3.2) and thus to supranormal elevation of testosterone levels, with a consequent increase in availability of substrate for aromatization (Figure 3.3). If androgens positively regulate aromatase expression, as various studies suggest (Simpson et al., 1997), further elevation of estrogen levels will occur. Thus, the overall "estrogenic" activity of PCBs, DDT, or phthalates *in vivo* could not be predicted from measurement of their activity in any *in vitro* estrogen screening system. Some estrogens are agonists in one tissue and antagonists in another (e.g., tamoxifen, raloxifene), and emerging understanding suggests that the same will hold true for androgens. Although the basis for these differences is not understood completely, it is clear that tissue-specific expression of co-activator proteins (or adapters) is involved and, in the case of estrogens, whether ER-α and/or ER-β is expressed. As some co-activators may be shared between various members of the steroid receptor superfamily, action of one member might alter availability of co-activators to interact with androgen- or estrogen-receptor complexes. Alternatively, nonreproductive hormones might regulate the expression of these co-activators. Although these possibilities are speculative, the insights afforded by tamoxifen, raloxifene, and other emerging SERMs (Cosman and Lindsay, 1999) emphasize the unpredictable pathways by which sex steroid hormone action can be altered.

Based on the considerations detailed above, it is clear that chemicals should be tested for their "reproductive" activity (i.e., the ability to alter the development or the function of the reproductive system) rather than just for their sex steroid activity in *in vitro* tests systems if the objective is to establish whether or not the compound in question is a reproductive endocrine disruptor.

3.12 Modes of Action and Phenotypic Outcomes of EDC-Related Developmental and Reproductive Toxicities

3.12.1 Scope of Survey

This section presents several selected examples of the mechanisms of action of several well-characterized EDCs that have been shown to produce adverse effects *in vivo* on reproductive function and development. As expected from the normal functioning of endocrine systems, the cellular and molecular mechanisms of endocrine disruption are not limited to receptor binding and include, for example, inhibition of hormone synthesis, metabolism, and transport. Figure 3.6 displays some of the mechanisms of steroid hormone action, as an example, and provides key steps in the process where EDCs have been shown to alter endocrine function. Other sites in the signal transduction process are also likely to be susceptible to disruption by anthropogenic chemicals. This section highlights the cellular and molecular mechanisms of action and toxicological impact on the developing reproductive system using selected examples of steroid receptor agonists and antagonists, steroid synthesis modifiers, and AhR agonists. When available, data from nonmammalian species are also presented. EDCs typically alter reproductive development by more than one mechanism such that several target organs are impacted, although not necessarily at the same dose or same precise stage of life. It is critical to recognize the limitations of *in vitro* tests, as the multiple endocrine and nonendocrine effects of a toxicant can only be interpreted in a meaningful and comprehensive fashion *in vivo*.

Considerable homology exists in the endocrinology of vertebrates; hence, toxicants that alter endocrine function in one species are likely to produce adverse effects in another. However, there are significant differences between some species in endocrine function that warrant consideration for further interspecies extrapolations. Although the hormones, hormone synthesis, and their receptors are highly conserved, the role of specific hormones in reproductive function and development can vary greatly. Additionally, significant differences in metabolism of EDCs can result in marked species differences in responses to these chemicals.

3.12.2 AR-Mediated (Anti)Androgens

3.12.2.1 Vinclozolin. It is generally assumed that mammals possess a single AR, as evidenced by the complete phenotypic sex reversal displayed in humans with androgen insufficiency syndrome as a consequence of a single base substitution in the AR (Quigley et al., 1995). Vinclozolin is a dicarboximide fungicide with AR antagonism. Of the EDCs, the cellular and molecular mechanisms of action of the antiandrogenic fungicide vinclozolin are one of the most thoroughly characterized. Vinclozolin metabolites M1 and M2 competitively inhibit the binding of androgens to the mammalian

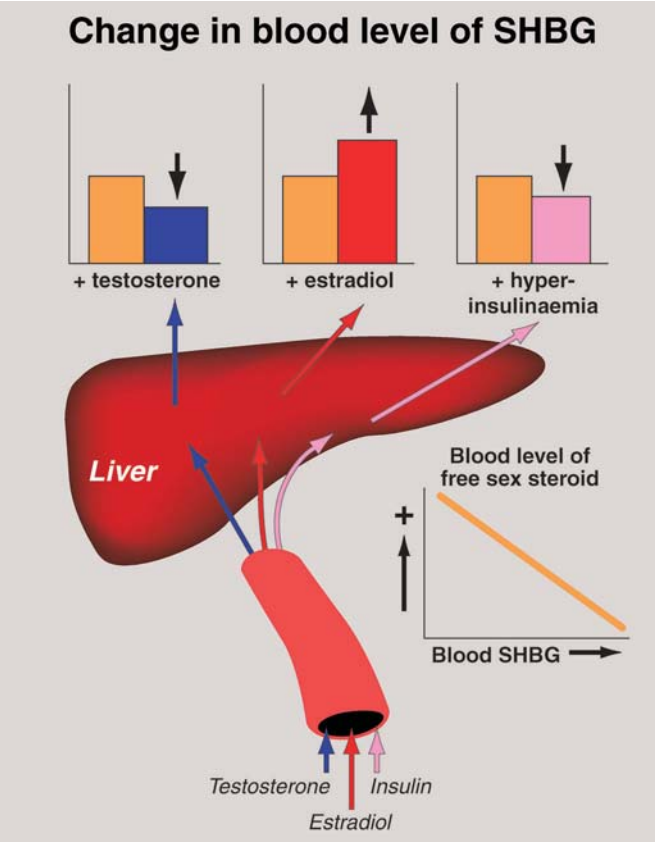

Figure 3.5 Diagrammatic illustration of one potentially important example of cross talk between endocrine axes in the human with particular relevance to endocrine disruption. In humans and some other mammals, the sex steroids testosterone and E_2 circulate in the bloodstream bound to SHBG and are thus not freely available to target cells. Variation in the levels of SHBG (produced by the liver) will alter the biological activity of testosterone and E_2 without altering their production. Perhaps not surprising, SHBG production is itself regulated by testosterone (negatively) and E_2 (positively). However, via cross talk, elevated levels of insulin also lead to suppression of SHBG production and, as a consequence, will increase the level of biologically active (= not bound to SHBG) testosterone and E_2. Moreover, because testosterone binds more strongly to SHBG than does E_2, a decrease in SHBG levels leads to a preferentially greater rise in bioactive testosterone and thus alters the androgen/estrogen balance. Factors that lead to elevation of blood insulin levels, such as a sugar-rich diet, can therefore potentially alter levels and actions of the sex steroids on target tissues.

AR. M1 and M2 also inhibit DHT-induced transcriptional activity in cells transfected with the human AR. Kelce et al. (1997) demonstrated that vinclozolin treatment altered gene expression *in vivo* in an antiandrogenic manner. In contrast to the binding to the AR, neither vinclozolin nor its antiandrogenic metabolites display affinity for the ER, although they do have weak affinity for the progesterone receptor. Vinclozolin, M1, and M2 do not inhibit 5α-reductase activity *in vitro*, the enzyme required for the conversion of testosterone to the more active androgen DHT (Kelce et al., 1994). A comparison of the *in vivo* and *in vitro* dosimetry data with the biological effects of vinclozolin reveals that when M1 and M2 concentrations in maternal serum approach their respective K_i values for AR binding, male offspring are malformed (Kelce et al., 1994; Monosson et al., 1999).

The ability of M1 and M2 to inhibit AR-dependent gene expression has been demonstrated both *in vitro* and *in vivo*. In addition, vinclozolin inhibits growth of androgen-dependent tissues in the castrate-immature-testosterone-treated male rat, a further demonstration of its antiandrogenic action *in vivo*. The drug flutamide is metabolically activated to hydroxyflutamide, which is similar in structure to the vinclozolin metabolite M2 and exhibits endocrine activity that is nearly identical to vinclozolin or M2, respectively (Imperato-McGinley et al., 1992; Gray et al., 1994; Kelce et al., 1995).

In addition to their antiandrogenic effects on the reproductive tract, vinclozolin and flutamide alter reproductive function at the level of the hypothalamic-pituitary axis. Oral treatment with vinclozolin (30–100 mg/kg/day; Monosson et al., 1999) or flutamide causes elevations of serum LH and testosterone levels and Leydig cell hyperplasia. In contrast to vinclozolin and flutamide, treatment with *p,p´*-DDE (Kelce et al., 1995) or MXC (Gray et al., 1989, 1999c), which are also antiandrogenic, fails to induce any significant change in serum LH or testosterone levels. A wide variety of antiandrogenic teratogenic effects in male offspring are noted following late gestational exposure to dose levels of vinclozolin in the range of 3–200 mg/kg/day (Gray et al., 1994, 1999b). These include a femalelike AGD, retained nipples, cleft phallus with hypospadias, suprainguinal ectopic testes, vaginal pouch, epididymal granulomas and small or absent accessory sex glands and delays in preputial separation. In a study of the low-dose effects of vinclozolin, pregnant rats were exposed to between 3 and 100 mg/kg/day from gestation day 14 to postnatal day 3. Even the lowest dose group (3.125 mg/kg/day) produced significant effects on AGD and retention of nipples in male offspring. Malformations of the male reproductive tract were observed at 50 and 100 mg/kg/day. Even though all of these end points (reduced AGD, retained nipples, effects on accessory sex gland weight, hypospadias, epididymal agenesis) are believed to be elicited by interference at the level of the AR, they display a wide variety of effective dose levels producing statistically significant changes. Some of these changes do not exhibit an obvious threshold in the range of the experimental dose levels. Multigenerational studies are essential to detect subtle antiandrogenic effects on male reproduction, and a failure to utilize the new testing guidelines (which incorporate new antiandrogenic measures) could yield NOAELs at least an order of magnitude too high.

Dermal exposure of adolescent rabbits to 100 mg/kg of vinclozolin for 2 months resulted in reduced accessory gland weights, but sperm counts were significantly elevated. The authors suggested that the antiandrogenic effects may have blocked the negative feedback of testosterone on the hypothalamus or pituitary allowing for increased gonadotropin release (Moorman et al., 2000).

3.12.2.2 Other AR antagonists. Several other toxic substances have been shown to display AR-antagonist activity, including the DDT metabolite DDE (Kelce et al., 1995; Gray et al., 1999a; You et al., 1998, 1999a, 1999b), the MXC metabolite HPTE (Gaido et al., 1999; Maness et al., 1998), the organophosphate fenitrothion (Tamura et al., 2001), and the dicarboximide fungicide procymidone (Ostby et al., 1999). Linuron is a urea-based herbicide that displays weak affinity for the AR, but the effects induced in male offspring indicate that it may alter mammalian sex differentiation via more than one mechanism of action (Gray et al., 1999a; Lambright et al., 2000; McIntyre et al., 2000). In this regard, the phytoantiandrogenic drug permixon, used clinically for prostate problems, appears to bind AR and inhibit steroid hormone synthesis as well (Carilla et al., 1984; Bayne et al., 1999; Plosker and Brogden, 1996). Tris(4-chlorophenyl)methanol is a global contaminant of unknown origin that is structurally related to DDT, has binding affinity for the AR comparable to *p,p´*-DDE. However, it has not demonstrated antiandrogenic effects *in vivo* when sexually mature rats were exposed for 28 days at doses up to 100 ppm in the diet (Foster et al., 1999).

3.12.2.3 AR-mediated effects among other vertebrates. Sexual dimorphisms in fish have been demonstrated to be affected by both androgens and estrogens (Ankley et al., 1998). For example, Smith (1974) demonstrated that the formation of breeding tubercles and a mucus-secreting dorsal pad in the fathead minnow is inducible by 17α-methyltestosterone. When aromatizable (testosterone and 17α-methyltestosterone) and nonaromatizable (11-ketotestosterone and 17α-methyl-DHT) were administered to newly hatched genotypic female chinook salmon for 2 hours, dose-dependent sex reversal was observed, with the synthetic and nonaromatizable forms more potent than the natural or aromatizable forms, thus indicating a role for aromatase early in development (Piferrer et al., 1993). Predominantly male populations of tilapia can be produced on a commercial scale by feeding androgens to fry. Administration of 25 mg of trenboline acetate for 28 days to sexually undifferentiated *Oncorhynchus aureus* resulted in 98% phenotypic males (vs. 55.7% males in the control group; higher exposures yielded lower percentages of males, presumably due to the less potent antiestrogenic activity (Galvez et al., 1996). In similarly treated channel catfish, Davis et al. (2000) found evidence that adults were lighter and shorter and had smaller gonads and GSI and lower plasma testosterone as adults than did control males. When the reproductively mature fathead minnows were exposed to methyltestosterone for 21 days, a decrease in plasma concentrations of sex steroids and adversely affected gonadal status (as evidenced by relative weight and histopathology) was observed in both sexes (Ankley et al., 2001). The androgenic nature of methyltestosterone was clearly evidenced by masculinization of exposed females. Vitellogenin induction was observed in both sexes, probably as result of aromatization of the administered androgen. Although mammals are believed to possess a single AR (Quigley et al., 1995), some piscivorous species have two ARs, termed AR1 and AR2 (Sperry and Thomas, 1999a, 1999b). AR1 in the brain displays binding affinities for ligands quite distinct from AR2, which has similar ligand affinities to mammalian AR. AR2 has been shown to bind *p,p´*-DDE and vinclozolin metabolites M1 and M2 (Sperry and Thomas, 1999a), demonstrating the homology of AR function *in vitro* among diverse species of vertebrates. *In vivo*, vinclozolin treatment induces intersex in the medaka (*Oryzias latipes*), which displays a mammalian-type sex differentiation (Koger at al 1999). In contrast, Makynen et al. (2000) did not obtain sex reversal in the fathead minnow with vinclozolin

treatment. This may be related to several factors, including a lack of metabolic activation of vinclozolin (Makynen et al., 2000) and the undefined role for androgens in the sex differentiation process. However, in contrast to the results cited above, M1 and M2 did not bind AR in this species (Makynen et al., 2000).

Takeo and Yamashita (1999) described two distinct rainbow trout cDNA clones, which were designated rtAR-α and rtAR-β, that contain the entire AR coding region. Comparison of the predicted amino acid sequence of rtAR-α to that of rtAR-β revealed 85% identity. Despite this high homology, rtAR-α activated transcription of an androgen-responsive reporter gene in cotransfection assays, but rtAR-β did not, suggesting that rainbow trout contains two distinct isoforms of ARs whose functions differ.

Ikeuchi et al. (1999) identified 11-ketotestosterone (a potent androgen in teleosts) as the spermatogenesis-inducing hormone of the Japanese eel and cloned its receptor (eAR1) cDNA from eel testis, and also reported that they cloned a second type of AR (eAR2) from the eel testis, encoding 797 amino acid residues. The amino acid sequence of eAR2 shows high homology with other ARs, including eAR1, in the DNA-binding (98–88%) and ligand-binding (59–85%) domains, whereas the other domains show low homology. In transient transfection assays of mammalian cells, the eAR2 displayed androgen-dependent activation of transcription from the androgen-responsive murine mammary tumor virus promoter. Tissue distribution of its mRNA differed from that of eAR1. The degree to which the differences in amino acid sequences in the ligand binding domain between ARs of lower vertebrates and mammalian AR affect binding of natural and synthetic ligands remains to be determined. Although it is clear that differences in overall sequence homology of this region exist, it appears that the amino acids in the binding pocket of the human AR that result in loss of function when mutated (Quigley et al., 1995) are more highly conserved in the fish ARs than are other amino acids in this domain.

3.12.2.4 Other sites of action of antiandrogens: liver and adrenal. Although vinclozolin and its metabolites do not bind the glucocorticoid receptor (Kelce et al., 1994), vinclozolin treatment has been shown to alter the pituitary-adrenal axis in several mammalian species, including rats (Monosson et al., 1999) and dogs. Indeed, the former no adverse effect level (NOAEL) was established on the effects of vinclozolin on the canine adrenal gland. The effects of vinclozolin (Monosson et al., 1999) and flutamide (Wada et al., 1999; Migliari et al., 1999) on liver function are also noteworthy. Although the mechanism of action for the hepatic effects of these antiandrogens has not been elucidated, androgens and antiandrogens acting via the AR are known to alter several aspects of liver growth and metabolism. In particular, cyproterone acetate, a drug that has antiandrogenic, progestational, and antiglucocorticoid activity, also acts as a liver mitogen (Kasper et al., 1999). The mechanisms of action of vinclozolin and flutamide on the liver warrant study, as these chemicals induce adverse effects with long-term treatment (flutamide-induced morbidity due to liver failure and vinclozolin-induced liver tumors in male rats).

3.12.3 ER-Mediated Estrogens

3.12.3.1 Overview. The ability of pesticides to act as estrogen agonists, inducing a uterotropic response, has been known for over 30 years (Bittman et al., 1968), and the estrogenicity of anthropogenic chemicals, for example, bisphenol A and DES, were first described in 1938 (Dodds and Lawson, 1938). Many estrogens have been identified using *in vitro* assays (e.g., ER binding, breast

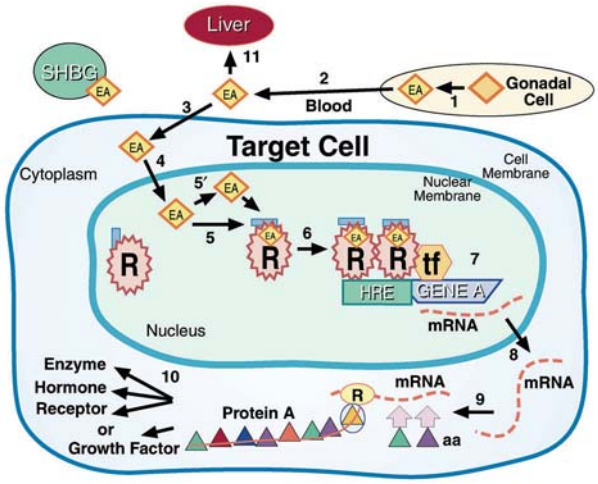

Figure 3.6 - Schematic diagram depicting several key steps in steroid hormone action that may be sensitive to disruption by environmental chemicals:

(1) Steroid hormones (EA) such as E_2, testosterone, and progesterone are synthesized in the gonadal cells. Inhibitors of CYP450 enzymes, including drugs and pesticides, act here.

(2) Hormones are secreted in the blood from the gonad and are available to the cell through diffusion or may be transported bound to SHBG. The degree of free and bound hormone depends upon several factors, but bound hormone disassociates from SHBG at a rate that depends on the binding affinity of the steroid for the SHBG protein. Toxicants could alter SHBG levels, and it is reported that some hormone mimicks do not bind SHBG as well as the natural ligands do, making them more available both to the target cell and for liver metabolism.

(3) The steroid hormone diffuses into the cell.

(4) The hormone diffuses into the perinuclear region, where unoccupied receptors (R) are located.

(5) The hormone, or hormone mimic, binds the receptor. Many xenobiotics have been shown to bind ER or AR. In some cases, the chemical secreted into the blood is a prohormone, that is metabolized in the cell (5′ to the active hormone. For example, in some tissues, testosterone is metabolized by aromatase to E_2, whereas in others the enzyme 5α-reductase converts it to DHT. In some tissues, such as muscle, testosterone itself is the active hormone. Some EDCs inhibit the activation of the prohormone in the target tissue.

(6) The receptor (R), now bound to a natural or synthetic ligand, undergoes a conformational change, exposing key protein binding sites, and forms homodimers.

(7) The homodimers, accumulate transcriptional factors (tf), forming a transcriptional complex, which binds to specific sequences on the DNA of hormone-dependent genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA). Some antihormones interfere with DNA binding.

(8) mRNA is transported out of the cell into the cytoplasm.

(9) In association with amino acids (aa) bound to specific tRNAs (the thick arrows) and ribosomes, proteins (the circles on a "string") are synthesized from the mRNA template.

(10) The protein, a marker of endocrine action, could be an enzyme, a protein hormone or growth factor, or a structural component of the cell. An example of a hormone-dependent marker is vitellogenin, an estrogen-sensitive protein produced by oviparous vertebrates.

(11) In some cases, toxicants disrupt endocrine function by altering liver function, either increasing or decreasing metabolism of the hormone, such that serum levels are altered. For example, some PCB stimulate metabolism of T_4, dramatically reducing serum T_4 levels. Several pesticides have been shown to stimulate the liver and reduce serum steroid hormone levels.

cancer cell proliferation, and transcriptional activation), and several also display estrogenic activity *in vivo,* including MXC, chlordecone, octylphenol, nonylphenol, bisphenol A and B, phytoestrogens (genistein), ethynyl estradiol, and fungal mycotoxins (zearalenone). Other chemicals that have shown evidence *in vitro* of estrogenic activity have not shown similar evidence in *in vivo* systems, and caution is warranted in interpreting *in vitro* results without *in vivo* confirmation. *In vitro*, some estrogens, such as bisphenol A and E_2, have been reported to interact in an unanticipated manner, with bisphenol A antagonizing some of the effects of E_2 (Gould et al., 1998). Phytoestrogens present in a variety of plants such as soy (isoflavanoids) and berries, fruits, grains, vegetables, and nuts (lignans) represent another source of exposure to estrogenic chemicals (reviewed in Whitten and Patisaul, 2001). Binding studies show that isoflavanoid phytoestrogens are high-affinity ligands for ERs, especially ER-β, but have lower potencies *in vitro* cell-based assays. *In vivo* data indicate that phytoestrogens have a wide range of biologic effects at doses and plasma concentrations seen with normal human diets. *In vivo* data effects have been reported for bone, ovary, pituitary, vasculature, prostate, and serum lipids. Effective doses in humans (0.4–10 mg/kg/day) are generally lower than those causing effects in rodents (10–100 mg/kg/day), although careful pharmacokinetic comparisons of circulating dose are not available to truly compare the species sensitivity.

3.12.3.2 MXC: an estrogenic and antiandrogenic pesticide. The estrogenic pesticide MXC is still in commercial use. This DDT derivative usually does not bioaccumulate because it can be metabolized by some species more readily than the metabolites of DDT. MXC provides an example of the multiplicity of EDC action because it is an ER-α agonist, an ER-β antagonist (Maness et al., 1998; Gaido et al., 1999), and an AR antagonist. *In vivo*, MXC displays estrogenic ER-α–mediated activity in many tissues, including the uterus, vagina, brain (behavior), and bone, but not in the hypothalamic-pituitary axis. MXC treatment failed to induce hyperprolactinemia, inhibit LH, or induce pituitary tumors in the rat after long-term high-dose treatment (Gray et al., 1988, 1989, 1999c). In the adult and pubertal male rat, MXC antagonizes the effects of androgens, which may indicate that MXC metabolites are inhibiting testosterone and DHT-induced gene expression and tissue growth and differentiation, as some of these tissues lack ER. Many natural and anthropogenic estrogens display affinity for AR, acting as AR antagonists and agonists in *in vitro* assays (Danzo, 1997; Baker et al., 1999), albeit often at high concentrations.

MXC itself is weakly active or inactive *in vitro* in ER binding and transcriptional activation assays. Purer forms of MXC (>99%) are inactive compared with less pure MXC (>95% pure) (Bulger et al., 1978a, 1978b). MXC is metabolically activated to several monohydroxy and dihydroxy metabolites that display estrogenic activity. One of these, HPTE also is a relatively potent AR and ER-β antagonist (Maness et al., 1998; Gaido et al., 1999) as well as an ER-α agonist.

Treatment of adult male rats with MXC alters fertility at very high doses by the inhibition of spermatogenesis (Gray et al., 1999c); lower dose levels (~25–200 mg/kg/day) reduce epididymal sperm reserves and seminal vesicle weight without affecting sperm production, testicular morphology, or serum testosterone levels. If treatment is administered at weaning, MXC will induce a number of effects in male offspring, including delays in puberty and reduced accessory sex gland weights. In the adult female, MXC induces effects more typical of an estrogen, with induction of lordosis. There were no effects on circulating LH or testosterone in the presence of delayed puberty, but there was an elevation in serum PRL. The lack of an effect on LH may be suggestive that, rather than working through the pituitary, the agent may have a direct effect on the reproductive tract. In longer term exposure, dosing of MXC for 10 months (200–400 mg/kg/day) to male rats delayed puberty by up to 10 weeks, reduced fertility, and altered reproductive behavior but did not mimic the chronic sustained effects of E_2 given via Silastic implants. In a study in which MXC was given to female rats by gavage at 0, 5, 50, or 150 mg/kg/day for the week before and the week after birth, with the pups then directly dosed with MXC from postnatal day 7 (Chapin et al., 1997a), the high dose of MXC reduced litter size by approximately 17%. AGD was unchanged, although male prepuce separation was delayed at the middle and high doses by 8 and 34 days, respectively. High-dose males impregnated fewer untreated females; epididymal sperm count and testis weight were reduced at the high and top two doses, respectively. Female effects (vaginal opening, estrous cyclicity) were noted at 50 mg/kg/day and above.

3.12.3.3 Mechanisms of action of xenoestrogens in other vertebrates. Several of the xenoestrogens bind one of the fish ERs with affinity similar to that displayed for mammalian ER (Loomis and Thomas, 1999). Octylphenol, nonylphenol, bisphenol A, *o,p*-DDT, ethynyl estradiol, and MXC display estrogenic activity in lower vertebrates (i.e., fish and frogs). In avian and mammalian species, *o,p´*-DDT, but not *p,p*-DDT (Bitmann et al., 1968), induced growth of the female reproductive tract.

Some of these xenoestrogens induce gonadal intersex (Kloas et al., 1999), vitellogenin synthesis in males (Kloas et al., 1999; Lutz and Kloas, 1999), and hermaphroditism and estrogen-dependent sexual dimorphisms (Noriega and Hayes, 2000). The toxicants that induce estrogen-dependent changes in coloration in the African reed frog (*Hyperolius argus;* Hayes, 1998a; Hayes and Menendez, 1999) are remarkably similar to those that induce a uterotropic response in the female rat, indicating a high degree of homology in ER-α function despite the differences in ER sequence.

In contrast, the estrogenicity of MXC is likely to be as widespread throughout the animal kingdom because it will not be estrogenic in those lower vertebrate species that are unable to metabolically activate it. Hydroxylation of MXC is required for estrogenicity and for excretion; hence, these species that cannot metabolically active MXC have a tendency to bioaccumulate this pesticide to the same degree as DDT. In male catfish pretreated with MXC or BNF alone or in combination, pretreatment with MXC but not BNF significantly reduced rates of MXC biotransformation, and pretreatment with MXC/BNF followed by MXC significantly induced serum vitellogenin, whereas MXC alone did not significantly elevate vitellogenin, thus demonstrating in this species that MXC can elicit estrogenic activity despite diminished capacity to form estrogenic metabolites (Schlenk et al., 1998). In a short-term reproduction test with the fathead minnow (*Pimepehales promelas*) initiated with reproductively mature animals exposed for up to 21 days, MXC decreased plasma concentrations of one or more steroids (testosterone, 11-ketotestosterone, E_2) in both sexes and caused a significant induction of plasma vitellogenin in males (Ankley et al., 2001). A significant decrease in fecundity was also observed at the same concentration that induced vitellogenin (3.56 µg/liter). Continuous exposure of adult male sheepshead minnow (*Cyprinodin variegates*) to *p*-nonylphenol, MXC, or endosulfan for up to 42 days was observed to induce a dose-dependent increase in hepatic vitellogenin mRNA and plasma protein within 5 days of

exposure to all but endosulfan (Hemmer et al., 2001). The fact that MXC, but not its estrogenic metabolite HPTE, alters amphibian germinal vesicle breakdown, which is dependent upon cell-surface hydroxyprogesterone receptor activation, indicates that this effect is not mediated by the ER. As noted in section 3.8, recent studies have shown that a variety of xenobiotic chemicals, in addition the disrupting genomic steroid actions, can also interfere with the nongenomic actions of steroids (Thomas, 1999). The finding that low concentrations (100 nM, equivalent to 30–40 ppb) of the estrogenic compounds kepone and *o,p′*-DDE interfered with progestogen induction of meiotic maturation of Atlantic croaker oocytes *in vitro* provided initial evidence for this novel type of endocrine disruption (Ghosh and Thomas, 1995). Subsequently, disruption of oocyte maturation by estrogenic compounds was confirmed in an amphibian species, *Xenopus*, exposed to MXC (Pickford and Morris, 1999). In addition, kepone was shown to partially block the stimulatory actions of progestogens on sperm motility in Atlantic croaker (Thomas et al., 1998). Estrogenic compounds such as *o,p′*-DDT and nonylphenol have also been shown to exert rapid estrogenic (agonistic) actions on rat smooth muscle cells and on croaker testicular androgen production (Ruehlmann et al., 1998; Loomis and Thomas, 2000). Recently, direct evidence has been obtained that estrogenic compounds can disrupt nongenomic steroid actions by receptor-mediated mechanisms (Thomas, 1999). Competition studies have shown that these compounds bind to the progestogen membrane receptors on fish oocytes and sperm and to the estrogen membrane receptor in fish testes as well as disrupting nongenomic steroid actions in these tissues (Das and Thomas, 1999; Thomas et al., 1998; Loomis and Thomas, 2000).

When a genetically male population of the common carp (*Cyprinus carpio*) was exposed to 4-*tert*-pentylphenol, no effects on sexual differentiation or proliferation of primordial germ cells were evident following 3 days of exposure during the embryo-larval period. However, longer exposures, starting before and including sexual differentiation, induced the formation of an oviduct that was persistent upon returning to clean water. Exposure during these times (days 24–51 posthatch) reduced the number of primordial germ cells (Gimeno et al., 1997). Bisphenol A was observed to increase vitellogenin levels in male rainbow trout (*O. mykiss*) following 14 days of exposure to 500 µg/liter, whereas constant or decreasing levels were present at lower exposure levels. However, the ratio of responding to nonresponding animals indicated that levels as low as 70 µg/liter were effective. Average liver concentration at the 500 µg/liter exposure level was 4.36 µg/g (Lindholst et al., 2000).

A comparative study of 44 PCBs, 9 hydroxylated PCBs, and 8 arochlors binding to the cloned reptilian (*Anolis carolinensis*), recloned rainbow trout (*O. mykiss*), and human ER linked to the glutathione *S*-transferase protein found that only three PCBs (104, 184, and 188) effectively competed with E_2 for binding to the fusion protein from all three species. Data from the reptilian and humans were more similar to each other than to the rainbow trout receptor (Matthews et al., 2000). Five of the mono-*ortho* PCBs (58, 60, 68, 70, and 74) and 9 of 18 di-*ortho*-substituted congeners (18, 44, 49, 99, 101, 112, 128, 138, and 153) weakly interacted, and three others (41, 47, and 115) exhibited moderate binding with the rainbow trout receptor. Similarly, the 13 tri-*ortho* congeners interacted only with the rainbow trout ER. These data suggest that significant differences in relative affinity for ligands may exist across vertebrate steroid receptors.

3.12.4 Inhibitors of Steroid Hormone Synthesis

3.12.4.1 Overview. Several classes of fungicides have been developed to inhibit fungal membrane synthesis and growth by inhibiting specific CYP450 enzymes, especially 14α demethylation of lanosterol, in the sterol pathways. The process of steroidogenesis is sufficiently conserved that these chemicals can also inhibit mammalian steroidogenesis. There are several CYP450 enzymes in the steroid pathway, and the binding affinity for each varies from chemical to chemical. In general, however, at relatively high concentrations, these are nonspecific inhibitors of CYP450 enzymes. Hence, effects are not limited to the reproductive system and include adrenal and liver steroid metabolism in mammals and ecdysteroid synthesis in invertebrates (Schurmeryer and Nieschlag, 1984; Pepper et al., 1990; Williams et al., 2000).

3.12.4.2 Ketoconazole. The antifungal imidazole derivative ketoconazole inhibits various enzymes that belong to the CYP450-dependent mono-oxygenases in rodents and humans such as side chain cleavage of cholesterol and 11β-hydroxylase in the adrenal and 17α-hydroxylase and C_{17-20} lyase in rat and human testes (Schurmeyer and Nieschlag, 1984; Pepper et al., 1990). For example, human testicular mono-oxygenase activities *in vitro* are reduced by 50% by 3.1 µM ketoconazole. Side effects of the use of ketoconazole as a therapeutic antiandrogen include gynecomastia. However, the effects on steroidogenesis are not selective for the testis, with ovary and adrenal effects reported. Effects on adult Leydig cell function have been noted in humans and rodents *in vitro*. When administered to adult rodents, ketoconazole can have dramatic effects on fertility even after a single dose (Bhasi et al., 1986; Heckman et al., 1992; Waller et al., 1990). It is more difficult to observe effects on male reproductive development resulting from a decreased testosterone production, as other effects on ovarian and uterine steroid responses tend to interfere with pregnancy maintenance. Treatment of pregnant dams with ketoconazole is more likely to affect the ability to maintain pregnancy due to effects on ovarian progesterone synthesis resulting in abortion/litter loss that would preclude the observation of marked effects on the pups (Gray et al., 1999a).

3.12.4.3 Aromatase inhibitors. Aromatase CYP450 converts C19 androgens to aromatic C18 estrogens. A number of pharmaceutical agents have been developed that inhibit aromatase, which have been applied as treatment for postmenopausal breast cancer (Brodie et al., 1999). This P450 enzyme is highly conserved in a wide variety of tissues and many species, but the overall homology of the genes (as there is more than one gene) with other CYP450s is only about 30%. Hence, this enzyme is considered to be in a separate gene family within the overall superfamily. A consequence of the lack of sequence homology with other P450 enzymes in the steroid pathway, inhibitors of aromatase may display greater specificity than drugs such as ketaconazole. A yeast-based screening assay has been proposed to test for this activity (Mak et al., 1999). The induction of imposex in mollusks exposed to tributyl tin has been linked to inhibition of aromatase and subsequent estrogen deficiency and enhanced androgen levels. Several fungicides inhibit aromatase activity in mammals, resulting in infertility in both sexes. Fenarimol treatment inhibits male rat mating behavior, presumably by inhibiting the conversion of androgens to estrogens in the brain (Hirsch et al., 1987; Gray et al., 1998), and also inhibits parturition because of the critical role of E_2 near term in the induction of labor. The effects of fenarimol in mammals differ from those seen with ketoconazole, because fenarimol does not inhibit androgen production in male rat or progesterone synthesis during pregnancy.

Fenarimol also has been shown to inhibit ecdysteroid synthesis in invertebrates, whereas in reptiles aromatase inhibitors inhibit female gonadal sex determination (Williams et al., 2000).

Fenarimol caused a dose-related decrease in male fertility in Wistar rats, with the effect particularly evident in the anatomically normal progeny of dams treated with fenarimol throughout life, including gestation and lactation (Hirsch et al., 1987). Based on the observation that the infertility was associated with the absence of vaginal sperm at the time of mating, the effect appeared to be the result of an absence of male sexual behavior. Gray and Ostby (1998) subsequently described a dose-related decrease in male mating behavior of rats when fenarimol was administered daily from weaning through adulthood. These results suggest that fenarimol is acting centrally to decrease male sexual behavior by inhibiting the conversion of testosterone to E_2 in the brain. Consistent with a central effect, Hirsch et al. (1987) noted that fenarimol concentrations in the brain of neonates whose mothers were treated were three- to fourfold higher, and half-life was four times longer, than in other brain regions.

Exposure of genetic stocks of all female chinook salmon (*O. tshawytscha*) to fadrozole [5-(5,6,7,8-tetrahyroimidazo[1,5-α]-pyridin-5yl) benzonitrile monohyrochloride, or CGS 16949A], a nonsteroidal aromatase inhibitor, for a 2-hour period when the gonads were totipotent caused genetic females to develop as males. Resulting males had testes that were indistinguishable in both size and function from genetic males, and were fertile (Piferrer et al., 1994). When fadrozole was given to adult female coho salmon (*O. kisutch*), a lowering in plasma E_2 was observed, associated with an increase in plasma 17α,20β-P. Ten days after injection, 67% of the fish exposed to 10 mg of fadrozole/kg body weight had ovulated, in contrast to 0% in the control group (Afonso et al., 1999), indicating an advancement in oocyte maturation and subsequent ovulation. Administration of fadrozole to male coho salmon during sexual maturation inhibited secretion of E_2 by the brain and increased plasma 17α,20β-P, and treated males began spermiation earlier than control males. In addition, fadrozole-treated fish had higher levels of testosterone and 11-ketotestosterone than did controls within 4 days of injection (Afonso et al., 2000).

3.12.4.4 5α-Reductase inhibitors. 5α-Reductase is the enzyme responsible for the conversion of testosterone to DHT, a more potent AR agonist. DHT acts specifically to masculinize the external genitalia of the male. Finasteride is a 5α-reductase inhibitor used clinically to combat androgen-dependent prostate cancer and more widely as a treatment for hair loss in adult men. Although this is not an environmental contaminant, it is used as an example because DHT plays such a significant role in the development of the male reproductive tract, and the impact of its inhibition on male reproductive development, especially the prostate and genitalia, is profound. Following oral exposure to rats on days 6–20 of gestation (Imperato-McGinley et al., 1992), AGD was reduced at doses as low as 0.003 mg/kg/day, hypospadias was observed beginning at 0.1 mg/kg/day, and 100% of the offspring were affected at 100 mg/kg/day. There was a significant decrease in prostate size at 25 and 50 mg/kg/day, with no further decrease at higher doses. Unlike AR blockade with flutamide, finasteride did not totally abolish prostate differentiation or completely feminize the external genitalia, despite increasingly higher doses. These results suggest that testosterone can compensate for DHT to some degree at the level of the AR. Wolffian differentiation, however, was not affected by inhibition of DHT, demonstrating its testosterone dependency, but seminal vesicle growth was impaired. AR blockade can inhibit testicular descent more effectively than inhibition of 5α-reductase activity (Spencer et al., 1991). It is suggested that finasteride causes hypospadias by preventing the formation of the medial mesenchymal plate that is necessary for assisting the movement of the urogenital sinus from the base to the tip of the genital tubercle (Clark et al., 1993). Additionally, external genital abnormalities can be produced in male rhesus monkey fetuses when dams are exposed to an oral dose (2 mg/kg/day) of finasteride on gestation days 20–100. No external genital malformations were seen in similarly exposed female fetuses or in fetuses of either sex following daily intravenous exposure of up to 800 ng/kg/day over the same period of gestation (Prahalada et al., 1997).

3.12.4.5 Phthalates. Phthalates are a broad class of chemicals used as plasticizers in a number of manufacturing processes, and as discussed below, the developmental effects of several phthalates (e.g., the dibutyl and diethylhexyl esters) are exerted via alterations in testosterone-synthesizing ability of the fetal testes. The reproductive toxicity in adults of some phthalates has been well described. For example, DEHP has been shown to target the rat testis of adults and juveniles (Gray and Butterworth, 1980; Sjoberg et al., 1985). The mode of action of the testicular toxicity is via a metabolite (the monoester, MEHP), with the target cell in the testis being the Sertoli cell, although the precise biochemical interaction has yet to be identified (Heindell and Chapin, 1989; Heindell and Powell, 1992). Attention has also been focused on the endocrine-active effects of phthalates, including interactions with both estrogen and androgen action. Zacharewski et al. (1998) reported that DBP, BBP, and DHP weakly competed with E_2 for binding to the ER in competitive ligand-binding assays. In gene expression assays using MCF-7 cells transiently transfected with Gal_4-HEGO, and the Gal_4-regulated luciferase reporter gene 17m5-G-Luc, 10 μM DBP, BBP, or DHP exhibited 36%, 42%, and 20% activity, respectively, when compared with the 100% response observed with 10 nM E_2. Only BBP induced luciferase activity (32%) in HeLa cells stably transfected with Gal_4-HEGO and 17m5-G-Luc constructs and imparted minimal ER-mediated viability to the E_2-dependent recombinant yeast strain PL3 on selective medium. No significant responses were observed with the five other phthalate esters in any of the *in vitro* assays. *In vivo*, none of the eight phthalate esters reproducibly induced significant increases in uterine wet weight in immature ovariectomized SD rats treated with oral doses of 20, 200, or 2,000 mg/kg of phthalate ester. Treatment with phthalate esters at the same doses did not affect the degree of vaginal epithelial cell cornification in mature ovariectomized rats. These results indicate that only selected phthalate esters (i.e., DBP, BBP, and DHP) exhibit weak ER-mediated activity in some *in vitro* assays at high concentrations, but none of the eight phthalate esters elicited *in vivo* estrogenic responses based upon results obtained from uterotrophic and vaginal cornification assays. These results serve to raise caution in assessing the potential hazard of chemicals based solely upon results of *in vitro* experiments.

More significantly, some phthalates (e.g., DEHP, DBP, BBP, di-isonyl phthalate, but not DEP, DMP or DOTP) induce antiandrogenic responses in fetal males. For example, male rat pups exposed during sexual differentiation to DBP or DEHP exhibit malformations in androgen-dependent tissues although apparently by a non-receptor-mediated mechanism (Gray et al., 1999a, 2000). Importantly, because the critical window lies outside that of the traditionally defined "period of organogenesis," these effects have been missed in standard developmental toxicology studies (Ema et al., 1992, 1993, 1994; Tyl et al., 1988; Narotsky et al., 1995).

A DBP multigenerational study showed marked effects on fertility of rats in the F_1 generation compared with their parents in the F_0 generation, with fewer and smaller litters and a 50% decrease in sperm count. In addition, these F_1 animals showed numerous male reproductive tract malformations at the highest dose level tested (~660 mg/kg/day) that were not observed at comparable dose levels in the standard developmental toxicity studies (Wine et al., 1997). Mylchreest et al. (1998) examined the critical differences in the exposure period between the F_0 and F_1 generations, and exposed pregnant and lactating animals and then examined their offspring. A high incidence of epididymal malformations and decreased sperm count were found, as well as delays in preputial separation and decreases in AGD of the male pups. All of the reproductive tract malformations seen in the multigeneration study could be reproduced in this shorter exposure regimen, and no effects were noted in female offspring. In another multigeneration study, however, exposure to 250 mg/kg/day DBP (the lowest dose tested) induced malformations in both male and female F_1 rats (Gray et al., 1999a). By narrowing the exposure window to just late gestation (gestation days 12–21), Mylchreest et al. (2000) essentially reproduced their earlier findings and also reported an increased incidence of retained nipples in the male offspring. Thus, DBP had all the attributes of a classical AR antagonist in affecting reproductive tract development with a LOAEL of 100 mg/kg/day that is much lower than LOAELs and most NOAELs for other toxicities of DBP. Mylchreest et al. (1999) compared the effects of DBP to the AR antagonist flutamide and showed many similarities in the pattern of effects but also a number of differences in tissue sensitivity, with the epididymis being the prime target for DBP malformations, whereas the prostate was the major target for flutamide. Neither DBP nor monobutylphthalate interacted directly with the AR.

That these effects are not mediated via AR antagonism are supported by the findings that DBP and DEHP, as well as their monoester metabolites, do not bind mammalian (rat or human) AR (Mylchreest et al., 1999; Parks et al., 2000). Although the precise cellular and molecular site of phthalate action in the fetal male is unknown, the testis appears to be the primary target (Mylchreest et al., 1998, 1999; Gray et al., 1999a; Parks et al., 2000). Maternal DEHP and DBP treatments induced dramatic reductions of fetal testosterone synthesis (Parks et al., 2000) and fetal androgen levels (Mylchreest et al., 1999; Parks et al., 2000), and altered Leydig cell morphology and function were evident. The fact that the selective AR for toxicity of the phthalates during development appears similar to that for testicular toxicity in pubertal males may suggest that some commonality in the initial molecular event initiates these adverse outcomes. It appears that the mechanism of action of phthalate-induced toxicity is widespread throughout vertebrates. Developmental reproductive toxicity of the phthalates is observed in the guinea pig, ferret (Lake et al., 1976), rabbit (DBP; Veeramachaneni, 2000), hamster (i.e., MEHP), and several strains of rats and mice, including the PPARα-knockout mouse. The fact that PPARα-knockouts display testicular and renal lesions after DEHP-treatment demonstrates that this receptor, apparently involved in the toxicity of MEHP in the liver, is not required for the expression of these other forms of toxicity (Ward et al., 1998).

A fish multigenerational study, which exposed medaka to DBP at environmentally relevant concentrations, detected abnormal gonadal function in the F_1 by not the F_0 generation (Patyna et al., 1999), although it failed to induce estrogenlike responses in this species. DBP also has been shown to alter androgen-dependent tissues in developing anurans (Higuchi et al., 1999; Ohtani et al., 2000).

3.12.5 AhR Agonists: TCDD, PCBs, and PCDFs

This class of EDCs is responsible for many well-characterized reproductive and populations effects in fish and wildlife (Peterson et al., 1993). Because these observed effects are used as support of the endocrine disruptor hypothesis and are believed to be caused by TCDD and structurally similar, synthetic halogenated hydrocarbons, it is important to understand the mechanism of action, as it relates to the endocrine disruptor hypothesis (Birnbaum, 1994). Although the effects caused by TCDD can be classified as an effect on signal transduction, these effects are not covered by the narrow definition of receptor-mediated effects of steroid hormones. Overall, the evidence supports the hypothesis that most, if not all, TCDD effects are mediated through the AhR (Okey et al., 1994; Hankinson, 1995), a cytosolic receptor protein that was first discovered by Poland and Glover (1977). The AhR signaling transcription pathway is initiated by TCDD diffusion into the cell, where it binds with high affinity to the cytosolic AhR protein complex, which also includes heat-shock protein 90 (Hsp90) and a 38-kDa, immunophilin-related protein (Ma and Whitlock, 1997; Carver and Bradfield, 1997). The ligand binding activates AhR and stimulates the dissociation of AhR-associated proteins. The ligand-receptor complex is subsequently translocated into the nucleus, where it dimerizes with AhR nuclear translocator (ARNT; Hankinson, 1995; Probst et al., 1993). The heterodimers are capable of recognizing and binding DNA at the consensus sequence, GCGTG, of dioxin-responsive elements (Denison et al., 1989; Dong et al., 1996). This action either increases or decreases the transcription of target genes (Nebert et al., 1993; Schmidt and Bradfield, 1996), including CYP450 (CYP1A1, CYP1A2; Quattrochi and Tukey, 1989), NAD(P)H:quinone reductase (Favreau and Pickett, 1991), class 3 aldehyde dehydrogenase (Asman et al., 1993), and glutathione *S*-transferase (Paulson et al., 1990).

The ARNT protein also pairs with HIF-1α to regulate genes active in response to low oxygen stress (Guillemin and Krasnow, 1997; Semenza, 1994; Wenger and Gassmann, 1997). Regulated genes include Epo for erythropoiesis (Semenza, 1994), VEGF for angiogenesis (Forsythe et al., 1996; Goldberg and Schneider, 1994; Maxwell et al., 1997; Shweiki et al., 1992), and GLUT-1 for glucose transport (Semenza et al., 1994; Wenger and Gassmann, 1997). AhR, ARNT, and HIF-1α belong to basic-helix-loop-helix/PAS protein family and are found in representative organisms of all five kingdoms (Hahn, 1998). In addition to responding to low oxygen in the case of ARNT-HIFα heterodimers, PAS proteins are involved in development and differentiation (Nambu et al., 1991; Isaac and Andrew, 1996), regulation of circadian clocks (Huang et al., 1995; King et al., 1997), and steroid receptor signaling (Yao et al., 1993). The fact that ARNT null mice are not viable beyond day 10.5 of gestation provides additional evidence of the importance of this protein (Kozak et al., 1997; Maltepe et al., 1997). It is possible that exposure to TCDD and subsequent recruitment of ARNT through AhR may inhibit other signal transduction pathways depending on ARNT (Chan et al., 1999). Thus, through its ability to interact with multiple signal transduction pathways and to induce or inhibit a variety of gene products, AhR agonists are capable of inducing a wide spectrum of biological effects at a number of different life stages and in a variety of species. Some of these responses do not easily fit the traditional definition of endocrine-mediated effects. In this assessment, biological effects that correlated with AhR occupancy were therefore not considered sufficient to invoke an endocrine-mediated

mode of action. Instead, the criteria listed at the end of this chapter were used to determine whether Ah-mediated effects would be included in this review. Because such information is less available for studies in wildlife, more leeway was used in determining whether to include findings in wildlife than in humans.

Male rats exposed *in utero* to a single dose of 0, 0.05, 0.20 or 0.80 µg/kg TCDD on day 15 of gestation displayed reduced fertility, as well as delayed puberty and altered reproductive organ weights (Gray et al., 1997a). Growth and viability of the pups were reduced only at 0.80 µg/kg, eye opening was accelerated (all dosage groups), and puberty was delayed (at 0.20 and 0.80 µg/kg). Treated progeny displayed transient reductions in ventral prostate and seminal vesicle weights, and epididymal sperm reserves and glans penis size were permanently reduced. Ejaculated sperm numbers were reduced (45% in the 0.8 and by 25% in the 0.05 and 0.2 µg/kg dosage groups) to a greater degree than were cauda or caput/corpus epididymal or testicular (unaffected) sperm numbers. Female offspring from this treatment regimen showed a delay in vaginal opening at 0.80 µg/kg. A persistent vaginal thread was present in 27% of the progeny at 0.20 and 92% at 0.80 µg TCDD/kg (Gray et al., 1997b). These effects did not appear to result from abnormal ovarian function during prepubertal development; neither serum E_2 levels nor ovarian E_2 production was reduced in 21- or 28-day-old progeny of dams exposed to 1 µg/kg. In addition, partial to complete clefting of the phallus was displayed in TCDD-treated rats (10% at 0.20 and 60% at 0.80 µg/kg), and these dosage levels also increased the length of the urethral slit, increased distance from the urethral opening to the tip of the phallus, and decreased distance from the urethral opening to the vaginal orifice. Although fertility rates were normal, time to pregnancy was delayed by treatment with 0.80 µg/kg. When necropsied at 20 months of age, females from the TCDD-dose groups displayed histopathological alterations of the reproductive tract. Thus, TCDD affects reproductive tract development in ways both similar and different than estrogens and antiandrogens. Fetal levels of TCDD as low as 8–13 ppt are associated with these reproductive alterations (Gray et al., 1995a, 1995b, 1997a, 1997b; Hurst et al., 1998, 2000a, 2000b).

Following exposure to a single dose of 2 µg/kg TCDD on gestation day 11.5, both control and TCDD-treated F_1 females mated successfully with a control male; 20% of the F_1 treated females did not become not pregnant (Wolf et al., 1999). In addition, 38% of pregnant F_1 females from the TCDD group died near-term, and there were reductions in the numbers of implants in pregnant animals and pups born live in the treated group. In the F_2, survival through weaning was drastically reduced (15% treated vs. 78% for control) by TCDD treatment of dams on postnatal day 0. F_1 female hamster offspring exposed *in utero* to TCDD also displayed external urogenital malformations, with most females having complete clefting of the phallus. Thus, adverse effects of TCDD persisted through two generations (F_1 and F_2), even though the F_1 generation was only indirectly exposed during gestation and lactation.

Altered neurological development is another health outcome associated with prenatal exposure to TCDD in experimental animals. Mably et al. (1992) reported demasculinization and feminization of sexual behavior in male rats following maternal exposure. At the age when sexual behavior was tested, AhR-dependent hepatic CYP450 levels and ethoxyresorutin dehydroxylase activity were not different from controls, thus demonstrating that effects on sexual behavior were due to long-lasting effects of developmental exposure and disturbance of organizational effects of sex steroids. In this model, levels of ERs in different brain regions were measured. (Bjerke et al., 1994). Although the AhR receptor is present in the developing nervous system, its role, if any, in normal development is unknown, and there is as yet no direct evidence for an implication of the AhR in brain development.

3.12.6 Mechanism for *p,p´*-DDE–Induced Eggshell Thinning in Oviparous Vertebrates

During the 1960s and 1970s, when the pesticide DDT was in the North American environment at greater concentrations, populations of several sensitive bird species declined because of unsuccessful incubation of eggs due to abnormally thin egg shells (Cooke, 1973). Many of these species (e.g., the double-crested cormorant) have experienced dramatic population increases since the use of DDT was banned in the United States and environmental concentrations have subsequently declined (Ludwig, 1984). The eggshell-thinning effect of *o,p´*-DDT and its potent, stable metabolite *p,p´*-DDE in sensitive species is well known. Species that normally produce eggs with a chalky valerite cover, including pelicans, cormorants, shags, and gannets, can produce eggs with a much reduced or completely absent cover following DDE exposure (Gould, 1972; Cooke et al., 1976). In these species, the shell-forming process was most impacted by DDE toward its termination. Other birds such as the great black-backed gull (Cooke, 1979a, 1979b) and the gray heron (Cooke et al., 1976) show a general reduction in all shell layers following DDE exposure. Changes in mineral composition of eggshells following DDE treatment have seldom been investigated (Longcore et al., 1971).

Several possible mechanisms of DDE-induced eggshell thinning (which may vary among species) have been suggested (Cooke, 1973, 1979a, 1979b). However, many of the most popular avian laboratory species, including the domestic chicken and the Japanese quail, are insensitive to DDE-induced eggshell thinning (Scott et al., 1975). Although the mechanism of eggshell thinning has never been completely deduced, research in this area has focused mainly on one sensitive avian species (Lundholm, 1980, 1982, 1984a, 1984b, 1984c, 1985, 1988, 1993, 1994; Lundholm and Mathson, 1983; Lundholm, 1987; Lundholm and Bartonek, 1991, 1992). Suggested mechanisms have included 1) limiting the supply of calcium to the shell gland from the blood by either changing uptake, excretion, or transport (Peakall et al., 1975; Haynes and Murad, 1985; Taylor and Dacke, 1984; Hagmann, 1982); 2) decreasing carbonate availability for shell formation via inhibition of carbonic anhydrase (Bitman et al., 1970; Peakall, 1970a, 1970b; Pocker et al., 1971; Cooke, 1973; Miller et al., 1976; Eastin and Spaziani, 1978); and 3) altering steroid hormone receptors or function (Lundholm, 1985, 1988).

Currently, the leading hypothesis regarding the mechanism of DDE-induced eggshell thinning involves an inhibition of PGs by the shell gland mucosa. PGs play an important role in the control and regulation of reproduction in birds (Lundholm and Bartonek, 1992). PG synthesis is decreased by *p,p´*-DDE in duck shell gland mucosa, both in *in vitro* experiments and following *in vivo* exposure (Lundholm and Bartonek, 1992). PG synthesis was not inhibited by *p,p´*-DDT or *o,p´*-DDE in *in vitro* experiments, in keeping with the potency of these congeners in causing egg shell thinning. Additionally, indomethacin treatment reduced eggshell thickness. It has been hypothesized that a furosemide-insensitive, PG-stimulated HCO_3^- transport could be inhibited in the shell gland mucosa of DDE-treated ducks, but further experiments have not supported that hypothesis (Lundholm, 1994).

The mechanism of DDE-induced eggshell thinning has been suggested to be quite complex. Eggshell thinning is associated with a decreased quantity of calcium in affected eggs, and in the mallard duck the effect of DDE has been associated with a decreased transport of calcium from the eggshell gland mucosa to the lumen fluid. Treatment of birds with DDE has been associated with a variety of biochemical changes that could be related to changes in calcium transport. Many of these biochemical end points are interrelated, and it is difficult to determine which are the direct targets of DDE and which are merely co-influenced by its action. The situation is complicated by the fact that sensitivities to DDE-induced eggshell thinning vary among avian species, and hence different mechanisms might be causing eggshell thinning in different species, as evidenced by different gross eggshell defects.

Although egg shell thinning induced by DDE and related chemicals is one of the most cited examples of endocrine disruption in wildlife, given the multiple hypothesis regarding the mode of action, it cannot be stated with certainty that it is indeed an result of endocrine disruption. The strongest evidence for the linkage comes from the findings of altered PG biosynthesis in the mucosal gland.

3.13 EDC Modes of Action for Carcinogenesis—The Effect of Atrazine

Concern for the endocrine-disrupting effects of atrazine, a triazine herbicide, arose following the observation of increased incidence of mammary tumors in a chronic bioassay in female SD rats exposed to 400 ppm atrazine in the diet for 104 weeks. These tumors also appeared in control females but occurred earlier in the treated females. No other tumors were present in the treated SD female rats or in male SD rats or male and female Fischer 344 rats (Stevens et al., 1994; Thakur et al., 1998).

The finding of earlier-onset mammary tumors led to an investigation into the estrogenicity of atrazine, but (under equilibrium conditions) atrazine was not able to compete with E_2 for binding to rat uterine ERs. A weak competition was noted if the cytosols were preincubated at 25°C prior to incubation with the tracer (Tennant et al., 1994a). Somewhat conflicting results have been seen in other studies. Daily exposure of adult Fischer rats to 120 mg/kg for 7 days resulted in fewer treated females displaying normal estrous cycles, and the number of days in diestrous was significantly increased. Fertility was reduced in females during the first week after exposure, but pregnancy outcome was not affected in those that became inseminated (Simic et al., 1994). However, treatment of adult, ovariectomized SD rats with up to 300 mg/kg atrazine by oral gavage for 3 days did not result in an increase in uterine weight, nor were there increases in uterine progesterone levels, suggesting the lack of an estrogenic potential. When E_2 (2 µg/kg s.c.) was given in conjunction with 300 mg/kg or orally administered atrazine, there was a weak inhibition (~25%) of the uterotrophic response (Tennant et al., 1994b). In a similar study, immature female SD rats were dosed with 0, 50, 150, or 300 mg/kg atrazine by gavage for 3 days. Uterine weight was not increased, but decreases in uterine progesterone receptors and peroxidase activities were noted; however, when combined with E_2, no antiestrogenic effect of atrazine was noted on the uterus, including decreases in uterine progesterone receptor binding and uterine peroxidase (Connor et al., 1996). In this same study, atrazine did not affect basal or E_2-induced MCF-7 cell proliferation or display agonist or antagonist action against E_2-induced luciferase activity in MCF-7 cells transfected with Gal_4-regulated human ER chimera.

To further evaluate effects on reproductive function, female LE and SD rats that had been screened for regular 4-day estrous cycles received 0, 75, 150, or 300 mg/kg/day atrazine by gavage for 21 days. In both strains, atrazine disrupted the regular 4-day estrous cycles. For the LE rats, all dose levels were effective, whereas SD rats required a higher dose (150 mg/kg/day) for a longer time for this effect to appear. The increased time spent in vaginal diestrus was associated with elevated serum progesterone and low E_2 concentrations, indicative of a repetitive pseudopregnant condition. This hormonal condition was not considered by the authors to be conducive to the development of mammary tumors, although there was some indication of prolonged estrous at the lowest dose tested (Cooper et al., 1996).

The strain difference noted in the premature onset of mammary tumors (insensitive Fischer 344 rats vs. sensitive SD rats) has been attributed to differences in the normal aging of the reproductive tract in these strains (Eldridge et al., 1994; Stevens, et al., 1994; summarized in Chapin et al., 1996). Reproductive cycling in the female SD rat begins to decline in animals less than 1 year of age, presumably due to the loss of sensitivity of adrenergic neurons in the hypothalamus that control GnRH release to the pituitary. This loss of stimulation reduces FSH and LH release and ultimately delays ovulation. The delayed ovulation, in turn, allows prolonged exposure to estrogens and an effect evident as persistent vaginal cornification. In contrast, adrenergic neurons of female Fischer 344 rats do not seem to lose their sensitivity to estrogen stimulation, and regular cycling is maintained for a much longer time period. Rather, reproductive aging in the Fischer 344 is believed due to inability to control daily PRL surges, a prolonged activity of the corpora lutea, and a higher level of progesterone release. Hence, the endocrine milieu of the aging SD rat, but not the Fischer 344 rat, favors development of mammary tumors and helps explain the difference in incidence of spontaneous tumors as females of these strains age.

Consistent with an effect on central nervous system function, atrazine exposure beginning at weaning alters the development of puberty in both the male (Stoker et al., 2000) and female rat (Laws et al., 1996; 2000b). In the male, doses as low as 12.5 mg/kg/day beginning on postnatal day 23 delayed preputial separation. At postnatal day 53, ventral prostate weights, but not testes weights, were reduced in rats treated with 50 mg/kg/day. Female rats were somewhat less sensitive, as it required 50 mg/kg/day to delay vaginal opening, and 100 mg/kg/day altered estrous cycles in the first 15 days after vaginal opening. In addition, *in vitro* studies involving PC12 cells have suggested that atrazine inhibits the cellular synthesis of dopamine mediated by tyrosine hydroxylase, and norepinephrine mediated by dopamine β-hydroxylase and, as result, reduces the potential of the neuronlike cells to release norepinephrine (Das et al., 2000). How atrazine accelerates the neuroendocrine aging of the reproductive axis in the SD rat, however, has not been determined.

Atrazine may exert neuroendocrine effects in other vertebrates. Ovulated female Atlantic salmon (*Salmo salar*) release a priming pheromone in the urine (an F-type prostagladin) that is subsequently detected by the olfactory system of the mature male salmon and results in increased levels of sex steroids and expressible milt. Short exposure of atrazine to male parr significantly reduced the olfactory response to PG F_{2a}. In addition, similar exposures reduced their ability to respond to the priming effect of ovulated female salmon urine. Atrazine also had an additional effect upon the testes, modifying the release of androgens and suggestive of an additional mode of action in this species (Moore and Waring, 1998).

3.14 EDC-Related Modes of Action in Neurotoxicity

3.14.1 Overview

There is evidence of neurotoxicity for over 850 workplace chemicals (IPCS, 2001b), including metals, organic solvents, agrochemicals, polyhalogenated aromatic hydrocarbons, natural neurotoxins, and pharmaceuticals/drugs of abuse. Because the reproductive endocrine system is primarily regulated by the neuroendocrine system, these chemicals are potentially EDCs. However, even with the close interactions between the nervous and endocrine systems, it has generally proven difficult to elucidate primary modes of action from secondary manifestations, even for chemicals that have known potential to influence hormone action. Although the mechanisms by which endocrine disruptors influence the nervous system are largely unknown, it is clear that two different modes of interaction of hormones with neural function must be considered: 1) effects related to activational properties of hormones in adult organisms resulting in transient changes and 2) organizational effects on hormone-dependent processes during neural development that can result in permanent changes of neurobehavioral function, particularly sex-dependent and sexual-related behaviors. Both actions may involve specific hormone receptors, such as the estrogen or the AR, or may be due to modulations of receptors for neurotransmitters that are reported to be influenced by hormones. For instance, GABA receptors, muscarinic and nicotinic receptors, NMDA receptors, σ-receptors, and neuropeptide receptors are implicated in steroid hormone action as well as membrane receptors coupled to second messengers (Mensah-Nyagan et al., 1999).

To further complicate matters, the nature of the influence on neurotransmitters and whether it is endocrine mediated may differ even with compounds that are structurally closely related. For example, prenatal/neonatal exposure to the 3,4,3′,4′-tetrachlorobiphenyl resulted in elevated concentrations of dopamine in the frontal cortex and of dopamine and its metabolites in the substantia nigra, whereas exposure to 2,4,2′,4′-tetrachlorobiphenyl resulted in significant decreases in concentrations of dopamine in the frontal cortex and caudate nucleus. In both cases, the changes persisted into adulthood (Seegal et al., 1997). The study suggested that the reductions in brain dopamine concentrations were a consequence of PCB congener–induced inhibition of the synthesis of dopamine in concert with changes in cholinergic receptor function, whereas the persistent elevations in brain dopamine may be mediated by alterations in steroid hormone function during key developmental periods. Coplanar congeners, in addition to their ability to interact at the AhR, also alter estrogenic function, either by enhancing the metabolism of estrogens to hydroxy- and catecholestrogens (Gierthy et al., 1988) or by down-regulating ERs (Safe et al., 1991).

Although chemicals that alter neurotransmitter concentrations, such as PCBs, are likely to influence neuroendocrine function and ultimately reproduction, there are only a few reports on this potentially important mechanism of endocrine disruption. Reproductive impairment in Atlantic croaker exposed to Aroclor 1254 was associated with a dramatic decline in LH secretion and hypothalamic levels of 5-HT, a neurotransmitter that has a stimulatory influence on LH secretion (Khan and Thomas, 1998). A subsequent study showed that that the decline in 5-HT concentrations was due to inhibition of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis (Khan and Thomas, 2001). The decreased 5-HT activity after PCB exposure resulted in decreased hypothalamic concentrations of LHRH and its secretion, leading to the down-regulation of LHRH receptors on gonadotropes and a decreased LH response to LHRH stimulation (Kahn and Thomas, 2000). Moreover, a specific inhibitor of tryptophan hydroxylase, parachlorine phenylanaline, mimicked these neuroendocrine effects of the PCB mixture as well as the subsequent reproductive impairment, whereas cotreatment of the PCB-dosed fish with 5-hydroxytryptophane, which bypasses this biosynthetic step, reversed the PCB effects (Khan and Thomas, 2001). Neuroendocrine disruption associated with alterations of neurotransmitter function has also been reported in croaker after exposure to lead (Thomas and Khan, 1997).

The distinction between chemical exposures of the developing as opposed to the mature nervous system is of particular importance in the present context for both toxicological and neurobiological reasons. Both the nature and the adversity of outcome may depend on the time window during which chemical exposure occurs. Some hormones, such as sex steroids or thyroid hormones, are known to have a strong and strictly time-coupled organizational impact on brain development (Gray and Ostby, 1998). From a neurobiological point of view, disruption of organizational factors during development in general and, more specifically, during brain development is important because long-lasting or irreversible neurobehavioral changes later in life may be the consequence of such interactions (Tilson, 1998). For example, thyroid hormones are known to affect brain development a) by increasing the rate of neuronal proliferation in the cerebellum, b) by timing neuronal proliferation and differentiation, and c) by organizing the pattern of neuronal migration to specific brain areas (Porterfield, 1994). In humans, endemic cretinism caused by iodine deficiency, congenital hypothyroidism, or maternal hypothyroidism is associated with well-known neurological and behavioral deficiencies, such as mental retardation, deaf-mutism, speech disorders, or motor deficits (Porterfield, 1994). However, the degree of thyroid dysfunction is clearly critical.

In the adult organism, endocrine disruption of the reproductive system is considered as a possible cause of neurobehavioral alterations either if gonadal hormones are shown to be affected in association with changes in reproductive behavior and nonreproductive neurobehavior, or if sexually dimorphic nonreproductive behavioral changes following chemical exposure are reported without endocrine data. A direct toxic effect on hormone-receptor-expressing neurons and glial cells must also be considered. Alterations of hormone concentrations may be caused by cytotoxic effects on hormone-producing organs, resulting in impaired synthesis or release of hormones. For example, PCB exposure resulted in fine structural lesions in the thyroid and inhibition of proteolysis of thyroglobulin, thereby decreasing the release of T_4 (Collins and Capen, 1980). In addition, thyroid hormone levels are influenced by increase in hormone metabolism due to PCB exposure (Barter and Klaassen, 1992) and by blocking the binding sites for T_4 at its serum transport proteins, which causes enhanced clearance from serum and decreased availability to tissues (Brouwer and Van den Berg, 1986).

The question of which mechanism underlies the effect of putative endocrine disruptors on a given neurobehavioral function ultimately depends on what is known about the regulation of that function at the cellular and subcellular level. However, the events that constitute the basis for higher neural functions are far from being understood for many of the end points investigated. The following are two examples of experimental studies in which attempts were made to delineate possible mechanisms for chemical-induced alterations of neuroendocrine and neurobehavioral effects. However, these studies are only the first steps in the elucidation of the mechanisms.

3.14.2 Sexual Differentiation of the Nervous System

It is generally assumed that sexual differentiation of the brain in rodents depends on the activity of aromatase (CYP19), an enzyme that converts androgens to estrogens. Aromatase has been detected in all mammal brains so far examined (Lephart, 1996), but its role in sexual differentiation in other than rodent species remains to be proven. Aromatase is expressed in several brain areas, including the HPOA, stria terminalis, amygdala, and striatum. Its regulation appears to differ in different brain areas regarding androgen dependence and the developmental phase of maximum activity (Lephart, 1996; Lauber et al., 1997a, 1997b; Küppers and Beyer, 1998; Roselli et al., 1998). In the HPOA, a region with several sexually dimorphic nuclei (Cooke et al., 1998), a sharp peak of activity was found at the end of gestation that declines to basal activity within the first 5 days after birth (Lephart, 1996).

Maternal exposure to a mixture of PCBs, reconstituted according to the congener pattern found in breast milk and containing *ortho*-chlorinated and coplanar congeners, caused decreases in aromatase activity at birth of male rat pups together with an elevated sweet preference and reduced testes weights and testosterone levels in adult male offspring (Hany et al., 1999). Sweet preference behavior is more pronounced in female rats, suggesting that reductions in hypothalamic E_2 results in a more femalelike differentiation of the brain that in turn causes a feminization of behavior in adulthood.

3.15 EDC-Related Modes of Action in Immunotoxicity

The major function of the immune system is to defend against infectious agents and certain neoplastic cells. Various cell types and their soluble mediators execute the function of the immune system in finely tuned concert. The maintenance of homeostasis requires bidirectional communication between the neuroendocrine and immune systems. Most of the influence of the brain on the immune system is exerted by hormones released by the neuroendocrine system. Indeed, receptors for hormones have been detected on cells of the immune system, whereas receptors for cytokines have been detected in the endocrine glands and brain. It is also noteworthy that almost all lymphoid tissues are innervated, although the role of this neuroregulatory pathway is largely unknown (reviewed by Heijnen et al., 1991; Weigent and Blalock, 1995; Besedovsky and Del Rey, 1996; Johnson et al., 1997).

The HPA axis represents the major pathway in the communication between the central nervous system and the immune system. Synthesis of glucocorticosteroid hormone (cortisol in man) by the adrenal gland, induced by ACTH from the pituitary gland, results in suppression of immune responses. Other mechanisms are those mediated by the direct action of neuropeptides, such as opioid peptides (Van den Berg et al., 1991), on immune cells that are either stimulatory or inhibitory. For their communication, cells of the immune system carry receptors for a number of hormones, neuropeptides, and neurotransmitters, such as CRH, ACTH, PRL, β-endorphin, GH, and sex steroids. In addition, cells of the immune system produce inflammatory cytokines, specifically, tumor necrosis factor-α, IL-1, and IL-6, that may act as endocrine hormones of the immune system, produced at distant sites and acting upon the central components of the HPA axis and the sympathetic system.

Cortisol, the final effector of the HPA axis, has multiple and profound immunosuppressive effects. Histologically, the thymus is the first organ affected by this hormone. Cortisol affects production, traffic, and function of leukocytes; this often leads to lymphopenia and monocytopenia. In addition, monocyte chemotaxis, bactericidal activity, and T-lymphocyte proliferation can be inhibited by cortisol. Glucocorticoids also inhibit the production of many cytokines. In addition, glucocorticoids inhibit the expression of adhesion and adhesion receptor molecules on the surface of immune and other cells and potentiate the acute-phase reaction induced by cytokines, primarily IL-6.

PRL has been shown to regulate various aspects of the immune system. Hypoprolactinemia is associated with impaired lymphocyte proliferation and decreased production of macrophage-activating factors by T lymphocytes. The endogenous opioid peptides α-endorphin, β-endorphin, and γ-enkephalin are also produced in the pituitary gland. Endorphin receptors similar to those in the brain are present on spleen cells and probably several others types of leukocytes. β-Endorphin has been shown to enhance T-cell proliferation and IL-2 production. One biological activity of the thymus that is under neuroendocrine control is the secretion of thymic hormones (Savino and Arzt, 1999). The secretion of thymulin, a nonapeptide produced by thymic epithelial cells, is modulated by GH and PRL. The interaction between the pituitary and thymus is demonstrated by the immunodeficiency of the thymus-dependent immunity that occurs in mice following injection with antisomatotrope hormone serum.

Modifying influences on immune responses have also been reported for sex steroids. The balance between male and female sex hormones, E_2 and testosterone, influences the extent of immune responsiveness. In general, the male sex hormone testosterone is immunostimulatory. E_2 and synthetic nonsteroidal estrogenic compounds such as DES are potent suppressors of specific immunity: the effects observed in rodents include thymic atrophy, suppression of thymus-dependent cellular immune responses, acceleration of autoimmune diseases, suppression of natural killer cell activity, myelotoxicity, and stimulation of the mononuclear phagocyte system (Luster et al., 1984). Considerable changes are seen in lymphoid organs during pregnancy, and increased serum E_2 levels during pregnancy correlate with lymphopenia and suppression of cellular immunity (Clarke, 1984). By evaluation of steroidal and nonsteroidal compounds with varying degrees of estrogenicity, Luster et al. (1984) provided evidence that immunotoxicity correlated for the most part with estrogenicity.

In humans, both the neuroendocrine system and the immune system are immature at birth and fully develop at later stages in life. The immune system is highly sensitive to regulation by glucocorticoids in the human newborn, a period of life in which the capacity to generate a cortisol response is decreased, suggesting that this represents an adaptational response of the immune system that preserves the important regulatory effects of glucocorticoids on the immune system during this delicate developmental period (Kavelaars et al., 1996). This may imply that the neuroendocrine system may also be very sensitive to EDCs during ontogeny. Regarding the immune system, its susceptibility to toxic compounds is most evident during the perinatal period of life, as shown in laboratory animal studies with various compounds, including TCDD (Vos and Moore, 1974) and hexachlorobenzene (Michielsen et al., 1999).

These considerations show that there are potentially multiple endocrine pathways that may influence the function of the developing and mature immune system and that these may be targets for EDCs.

3.16 Basis for Attribution of Effects to Endocrine Disruption

The foregoing examples were cases in which the mode of action of an EDC has been generally well characterized in the laboratory setting in order to illustrate the types of interferences that are of importance and the variety of manifestations of adverse health outcomes that can occur. Some of the principles for defining cause-and-effects relationships that emerge from these examples, and that are appropriate to consider in the context of the rest of this review, are as follows:

(1) Ability to isolate the response to endocrine sensitive tissues in an intact whole organism
(2) Analysis of the response at multiple levels of biological organization—from phenotypic expression to physiology, cell biology, and ultimately molecular biology
(3) Direct measurement of altered hormone action (gene induction or repression), hormone release, hormone metabolism, or hormone interactions under the experimental regime in which the toxicologic outcome was manifest
(4) Dose–response observations that indicate the perturbance of the endocrine system is a critical response of the organism, and not the secondary result of general systemic toxicity
(5) Ability to compare resulting phenotypes with outcomes from exposures to known pharmacological manipulations
(6) Indication that there is differential sensitivity of certain life stages in which dysregulation of a particular endocrine system is known to have adverse health consequences
(7) Ability to restore the phenotype or toxicologic outcome via pharmacological manipulations that counter the presumed mode of action on the endocrine system in the intact organism
(8) Supporting data on endocrine activity from *in vitro* binding, transcriptional activation, or cellular response studies

Clearly, not all these components need be present in a single situation for a determination to be made that a chemical exposure has had an adverse health impact via modification of the normal functioning of the endocrine system. However, a collective weight-of-evidence approach is needed to classify the conditions under which the exposure is "endocrine disruptive." In Chapter 7 of this assessment, we extend the evaluation of cause and effect to natural populations, both human and wildlife, with particular emphasis on the presumed mechanistic basis for which a particular outcome is linked to particular exposure.