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DETAILED REVIEW PAPER ON THE STATE OF THE SCIENCE ON NOVEL IN VITRO AND IN VIVO SCREENING AND TESTING METHODS AND ENDPOINTS FOR EVALUATING ENDOCRINE DISRUPTORS

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No. 178

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FOREWORD

This Detailed Review Paper (DRP) was developed as a follow-up to the workshop on OECD countries' activities regarding testing, assessment and management of endocrine disrupters, which was held in Copenhagen (Denmark) on 22-24 September 2010 (see document No. 118 published in the Series on Testing and Assessment).

In 2010, the project was included in the Test Guideline workplan. It was led by the US, with the support of the European Commission (EC) and the Secretariat – the EC and the Secretariat led the development of the chapter on Endocrine Disrupters and the Epigenome, included in the annex of this DRP. The document was developed by consultants in close cooperation with an advisory group on testing and assessment of endocrine disrupters (EDTA AG). The outline of the DRP was discussed at the meeting of the EDTA AG in April 2011. The 1st draft DRP was then sent to the Working Group of National Coordinators of the Test Guidelines Programme and the EDTA AG for comments in July 2011. The draft was revised by the consultants on the basis of the comments received and a 2nd draft was developed and sent for WNT comments in autumn 2011. The 2nd draft DRP was also reviewed and revised at a meeting of the EDTA AG in December 2011. The draft chapter on Endocrine Disrupters and the Epigenome was circulated once more to the WNT after the December EDTA AG meeting, as its section on Recommendations (section 8 of the chapter) had been revised in depth by the EDTA AG.

The draft Detailed Review Paper was approved by the WNT at its meeting held in April 2012. It was declassified by the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology (hereafter Joint Meeting), on 7 August 2012. This document is published under the responsibility of the Joint Meeting.

Detailed Review Paper State of the Science on Novel *In Vitro* and *In Vivo* Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors

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Acronyms and Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
3,3',5-triClBPA	3,3',5-trichlorobisphenol A
17,20β-P	17,20β-dihydroxypregn-4-en-3-one
17,20β-S	17,20β,21-trihydroxypregn-4-en-3-one
ACTH	corticotropin
AhR	aryl hydrocarbon receptor
AND	androstenedione
ANSA	8-anilina-1-napthalenesulfonic acid
AOP	adverse outcome pathway
AR	androgen receptor
AR STTA	AR transactivation assay
ARNT	aryl hydrocarbon receptor nuclear translocator
ATPase	adenosine triphosphatase
AVP	arginine vasopressin
AVT	argonine vastocine
BDE	brominated diphenyl ether
BDE-47	2,2',4,4'-tetrabromodiphenylether
BDNF	brain-derived neutrophic factor
BFR	brominated flame retardant
BIAC	Business and Industry Advisory Committee
BKME	bleached kraft mill effluent
BMI	body mass index
BNF	β-naphthoflavone
BNST	bed nucleus of the stria terminalis
BPA	bisphenol A
BTEB	basic transcription element binding protein
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CARLA	co-activator-dependent receptor ligand assays
CAT	chloramphenicol actyltransferase
CBG	corticosteroid binding globulin
CBP/p300	CREB binding protein
CCD	charged couple device
CDCA	chenodeoxycholic acid
CG	chorionic gonadotropin
CHIP	chromatin immunoprecipitation
CHO	Chinese hamster ovary cells
CNS	central nervous system
CoR	co-repressors
CPT1	carnitine palmitoyl transferase 1
CREB	cAMP-responsive element binding
CRH	corticotropin-releasing hormone
CRH-BP	corticotropin-releasing hormone binding protein
CV	coefficient of variation

DBD	DNA binding domain
DBP	di-n-butyl phthalate
DEHP	di[2-ethylhexyl] phthalate
DES	diethylstilbestrol
DEX	dexamethasone
DHEA	dehydroepiandrosterone
DHEAS	DHEA sulfate
DHRA	9-*cis*-4-*oxo*-13,14-dihydroretinoic acid
DHT	dihydrotestosterone
DIDP	diisodecyl phthalate
DIT	di-iodothyronine
DMBPA	3,3'-dimethylbisphenol A
DNHP	di-n-hexyl phthalate
DnOP	di-n-octyl phthalate
DPSA	Differential Protease Sensitivity Assay
DRE	dioxin response element
DRP	detailed review paper
DUOX/ThOX	dinucleotide phosphate oxidase
DXA	dual-emission X-ray absorptiometry
EAT	estrogen, androgen, and thyroid
EDC	endocrine disrupting chemical
EDTA	Endococrine Disrupter Testing and Assessment
EE2	ethinylestradiol
EHA	2-ethylhexanoic acid
EMSA	*electrophoretic mobility shift assay*
ENCODE	Encyclopedia of DNA Elements
EPA	U.S. Environmental Protection Agency
ER	estrogen receptor
ERRγ	estrogen-related receptor γ
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
FXR	farnesoid X receptor
GABAergic	gamma-amino butyric acid
GHRH	growth hormone releasing hormone
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin-releasing hormones
GPR30`	G-protein-coupled receptor 30
GR	glucocorticoid receptor
GR_1	mineral corticoid receptor
GRE	glucocorticoid response elements
GFP	Green Fluorescent Protein
GST	glutathione S-transferase
GSUα	glycoprotein-hormone α-subunit
GVBD	germinal vesicle breakdown

H3K9me	3histone H3 lysine 9 trimethylation
HBCD	1,2,5,6,9,10-αHexabromocyclododecane
HCBD	hexabromocyclododecane
HHPS	hypothalamo-hypophysial portal system
Hnpc	human neural progenitor cell
HPA	hypothalamus-pituitary-adrenocortical
HPG	hypothalamo-pituitary-gonadal
HPI	hypothalamic-pituitary-interrenal
HPT	hypothalamo-pituitary-thyroidal
hrTPO	human recombinant TPO
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IHEC	International Human Epigenome Consortium
IL	interleukins
IP_3	inositol trisphosphate
IPS	induced pluripotent stem
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
K^+	potassium ion
LBD	ligand-binding domain
LC	locus caeculeus
LCA	lithocholic acid
LH	luteinizing hormone
LICA	ligand induced complex assay
LNG	levonorgestrel
LUMA	luminometric methylation analysis
LXR	liver X receptor
MBP	mono-n-butyl phthalate
MC_2R	melanocortin receptor
MCPA	2-methyl-4-chlorophenoxyacetic acid
MCT	monocarboxylate transporter
MCT8	monocarboxylate transporter 8
ME	malic enzyme
MEHP	mono-2-ethylhexylphthalate
MIT	mono-iodothyronine
MMGT	medaka multi-generation test
MMI	methimazole
mPR	membrane-G-protein-coupled gestagen receptor
MPS	massively-parallel sequencing
MR	mineralocorticoid receptor
MUFA	monounsaturated fatty acid
Na^+	sodium ion
NCoR	nuclear receptor corepressor
NGF	nerve growth factor
NIH	National Institutes of Health
NIS	sodium-iodide symporter
NR	nuclear receptor

OATP	organic ion transport proteins
OECD	Organization for Economic Cooperative Development
P_4	progesterone
PACAP	pituitary adenylate cyclase-activating peptide
PAH	polycyclic aromatic hydrocarbon
PAX8	paired box gene 8
PBB	polybrominated biphenyl
PBPK	physiologically based pharmacokinetic
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PGJ2	prostaglandin J2
PKA	phosphokinase A
PKC	protein kinase C
PLC	phospholipase C
PPAR	peroxisome proliferator activated receptor
pQCT	peripheral quantitative computed tomography
PR	progesterone receptor
PRL	prolactin
PTH	parathyroid hormone
PTU	propylthiouracil
PUFA	polyunsaturated fatty acid
PVN	paravocellular nucleus
PXR	pregnane X receptor
qPCR	real-time polymerase chain reaction
RAR	retinoic acid receptor
RIA	radioimmunoassay
RIC20	relative inhibitory concentration
RN	raphe nucleus
RU486	mifepristone
RXR	retinoid X receptor
SEXDAMAX	Sexual differentiation and metamorphosis assaywith *Xenopus*
siRNA	short interfering RNA
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SON	supraoptic nucleus
SRIF	somatotropin release inhibiting factor
SRC-1	steroid receptor coactivator-1
SRC-2	steroid receptor coactivator-2
STAR	steroidogenic acute regulatory protein
T3	thyroid hormone, triiodothyronine
T4	thyroid hormone, thyroxine
TBBPA	3,3’,5,5’-tetrabromobisphenol A
TBG	Thyroid hormone-binding globulin
TBTO	tributyltin oxide

TCBPA	3,30,5,50-tetrachlorobisphenol A
TCDD	tetrachlorodibenzo-*p*-dioxin
TCGA	The Cancer Genome Atlas
TD	thyroid disruptor
TDC	thyroid disrupting compound
TDS	testicular dysgenesis syndrome
TG	test guideline
TH	tyrosine hydroxylase
TIF2	transcriptional intermediary factor
TIQDT	T4 immunofluorescence quantitative disruption test
TMBPA	tetramethylbisphenol A
TMTU	N,N,N',N'-tetramethylthiourea
TPO	thyroid peroxidase
TR	thyroid hormone receptor
TRAP	tartrate-resistant acid phosphatase
TRE	thyroid response element
TRH	thyrotropin releasing hormone
TRHR	thyrotropin-releasing hormone receptor
TRIAC	T3 signaling agonist
TSH	thyrotropin stimulating hormone
TTF1	transcription termination factor 1
TTF2	transcription termination factor 2
TTR	transthyretin
Ucn	Urocortin
$V_{1a}R$	vasopressin-1
VDBP	vitamin D binding protein
VDR	vitamin D receptor
VDRE	vitamin D response element
VTG	vitellogenin
WHO	World Health Organization
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis

ABSTRACT

Increasing incidents of disorders such as obesity/diabetes/metabolic syndrome, reproductive dysfunction, and neuro-developmental abnormalities in some human populations have raised concern that disruption of key endocrine-signaling pathways by exposure to environmental chemicals may be involved. This Detailed Review Paper describes some endocrine pathways that have been shown to be susceptible to environmental endocrine disruption and whose disruption could contribute to increasing incidents of some disorders in humans and wildlife populations. Assays and endpoints are described that could be used in new or existing Organization for Economic Cooperative Development (OECD) Test Guidelines for evaluating chemicals for endocrine-disrupting activity. Endocrine pathways evaluated were the hypothalamus:pituitary:adrenocortical (HPA) axis, the hypothalamus:pituitary:gonad (HPG) axis, the somatotropic axis, the retinoid signaling pathway, the hypothalamus:pituitary:thyroid (HPT) axis, the vitamin D signaling pathway, and the peroxisome proliferator-activated receptor (PPAR) signaling pathway. In addition, the potential role of chemical-induced epigenetic modifications to endocrine signaling pathways, during sensitive windows of exposure, was evaluated as a mechanism of endocrine disruption, along with the examination of potential methods for assessing such disruption. This section is provided as an annex to the document (Annex 1). Potential targets of disruption along putative adverse outcome pathways associated with the signaling pathways were identified, along with assays that show promise in evaluating the target in a screening and testing program. Disruption of the HPA or retinoid X receptor signaling pathways could contribute to disorders of emerging concern, and adverse outcome pathways are well defined. However, assays for the assessment of disruption of these pathways are less well developed, and in some cases, are not specific to the pathway. Several new assays were described for the detection of disruption of the HPT axis. These assays may complement assays in the existing Test Guidelines and strengthen the adverse outcome pathway lineage. Assays for the detection of vitamin D signaling disruption and novel aspects of the HPG axis (membrane receptor signaling, progestin signaling) require further development and refinement prior to consideration for incorporation into Test Guidelines. Disruption of the somatotropic axis is likely to occur through disruption of other signaling pathways that cross-talk with the somatotropic axis. Disruption of the somatotropic axis may thus provide a more holistic view of the general integrity of the endocrine system. PPARs are involved in lipid and glucose homeostasis, inflammation, and aspects of development. The adverse outcome pathway for PPARγ is well established. Assays used to assess disruption of PPAR signaling are well developed, and many are suitable for incorporation into existing OECD Test Guidelines. In conclusion, OECD Test Guidelines could be modified to include new assays or the incorporation of novel endpoints into existing assays that would expand the repertoire of endocrine signaling pathways included in the screening and testing regimen.

INTRODUCTION

1. The endocrine system consists of an assemblage of ductless glands that secrete hormones directly into the blood or lymph, which regulate a wealth of biological processes.[1] The endocrine system is comprised of multiple pathways, or axes, each consisting of different groupings of organs and hormones with distinct regulatory functions. These pathways are intricately involved in organizational, or programming, events during fetal development, as well as in the maintenance of homeostasis in the adult organism. Mounting evidence has shown that aspects of the endocrine system are susceptible to perturbation by exogenous chemicals, resulting in the disruption of those processes under endocrine control. Evidence to date indicates that hormone nuclear receptors are a major target of endocrine disrupting chemicals (EDCs) because these receptors are designed to bind small, lipoidal molecules (i.e., steroid hormones), which can be mimicked by many environmental chemicals. These nuclear receptors, once activated by their ligand, regulate the transcription of target genes. Xenobiotics can disrupt normal nuclear receptor function by inappropriately activating the nuclear receptor (hormone receptor agonist) or by inhibiting the action of the nuclear receptor (hormone receptor antagonist). Some environmental chemicals also can disrupt normal endocrine function by altering circulating hormone levels. Accordingly, the World Health Organization (WHO) has defined an endocrine disruptor as an *exogenous substance or mixture that alters functions(s) of the endocrine system and, consequently, causes adverse health effects in an intact organism, or its progeny, or (sub)populations.*[2] In this detailed review paper (DRP), an EDC is defined as a chemical substance that meets this definition of an endocrine disruptor.

2. At the request of member countries and its Business and Industry Advisory Committee, the Organization for Economic Cooperative Development (OECD) established a Special Activity on Endocrine Disrupter Testing and Assessment (EDTA) in 1996. The objective of the Special Activity was to coordinate the development of Test Guidelines to detect endocrine disruptors and to harmonize risk characterization approaches for such chemicals. As a result, several Test Guidelines have been developed or are presently in development. These guidelines have been integrated into a Conceptual Framework that can be used to evaluate chemicals for endocrine-disrupting activity. The Framework (http://www.oecd.org/document/58/0,3343,en_2649_34377_2348794_1_1_1_1,00.html) organizes tests into five levels of complexity dealing largely with the ability of chemicals to disrupt estrogen, androgen, and thyroid (EAT) signaling processes and steroidogenesis. Level 1 consists of the compilation of all existing test data, physical-chemical properties of the chemical, and various model predictions of activity. Level 2 consists of *in vitro* screening assays that provide information on potential interactions between the chemical and specific endocrine target (e.g., receptors, enzymes). Level 3 consists of whole-organism screening assays that provide insight into chemical interactions with single selected endocrine mechanism(s) /_signaling pathways. Level 4 consists of whole-organism assays that provide data on adverse effects on endocrine-relevant endpoints. These assays provide insight into chemical interactions with multiple endocrine signaling pathways or endpoints. Level 5 consists of whole-organism assays that are designed to provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism.

3. This Conceptual Framework provides a rational, step-wise approach to evaluating chemicals for their ability to disrupt signaling pathways, with emphasis on EAT endocrine pathways. However, the EAT pathways represent three of many endocrine pathways, and recent evidence indicates that other endocrine pathways also are susceptible to the disrupting effects of environmental chemicals. Accordingly, the OECD recognizes the need to have Guidance Documents in place that also would serve to evaluate the effects of chemicals on non-EAT endocrine pathways. This DRP describes assays that have been used to detect endocrine-disrupting effects of chemicals on non-EAT pathways, atypical EAT pathways (e.g., estrogen signaling via membrane receptors), and neuroendocrine pathways. In addition, new approaches to assessing chemical effects on EAT pathways are discussed. The neuro-endocrine pathways discussed may function upstream to regulate the production of hormones that interact with nuclear receptors, or may

function through the production of peptide hormones, which contribute directly to endocrine signaling. (Note: The term *neuro-endocrine* is used in this document to denote both neuroendocrine and endocrine components to signaling pathways).

4. In 2007, the National Research Council published *Toxicity Testing in the 21st Century: A Vision and a Strategy*.[3] This document served to redirect the standard toxicity testing paradigm, which consists of a patchwork of disparate tests performed largely with animals, to a more organized approach that makes extensive use of *in vitro* assays to identify and characterize toxicity pathways. The authors argue that the use of *in vitro* tests, coupled with modeling approaches (e.g., physiologically based pharmacokinetic [PBPK] modeling), could reduce the time and expense of chemical toxicity characterization and would relegate the use of whole-animal studies, mainly to the validation of toxicity predictions. Adverse outcome pathways (AOP) have been used as a tool to formulate pathway linkages among molecular events and toxicity. An AOP is a conceptual framework that integrates molecular events initiated by exposure to chemicals or other physiologic stressor to adverse biological outcomes at relevant levels of biological organization (**Figure 1-1**).[4] In line with this emerging paradigm, assays described in this DRP are divided into *in vitro* screening assays designed to identify interactions of chemicals with specific components of toxicity pathways (OECD Conceptual Framework Level 2) and *in vivo* assays that would provide a more holistic evaluation of the chemical effects on endocrine signaling processes (OECD Conceptual Framework Levels 3–5). AOPs presented in the DRP are not meant to be definitive but are structured to define linkages between *in vitro* screening assays, which identify molecular initiating events, and *in vivo* toxicity tests that describe toxic events related to the initiating events. Readers are directed to Ankley et al.[4] for the discussion and presentation of detailed AOPs.

5. Interaction of EDCs with nuclear receptors stands prominent among the molecular events that initiate adverse outcomes. The nuclear receptor family has 48 functionally distinct members in humans.[5] In addition to the receptors involved in EAT signaling, hormone-activated nuclear receptors in vertebrates include the corticosteroid receptors (e.g., mineralocorticoid, glucocorticoid), retinoic acid receptor (RAR), retinoid X receptor (RXR), vitamin D receptor (VDR), and peroxisome proliferator activated receptor (PPAR). Ligands to some of these receptors (e.g., vitamin D binding to the VDR, retinoids binding to the RAR, fatty acids binding to PPAR) may not fit the conventional view of a hormone. Nonetheless, these ligands do fit the broad definition of a hormone as *a substance, originating in one tissue and conveyed by the bloodstream to another to effect physiological activity*,[1] and this document will address the pathways to which these hormones and receptors contribute and their susceptibility to disruption by environmental chemicals.

6. Members of the nuclear receptor family all share a common domain structure (**Figure 1-2**). The A/B domains are highly variable among the nuclear receptors, but contain a transcriptional activation function (AF-1) that is vital to receptor activity. The C or DNA-binding domain (DBD) is highly conserved among the nuclear receptors, containing two zinc finger motifs that are responsible for recognition of specific DNA binding sites. The D domain functions as a hinge between the DBD and the ligand-binding domain (LBD). The LBD or E domain contains a hydrophobic ligand-binding pocket, which provides specific ligand recognition to the receptor. The E domain mediates dimerization and ligand-dependent transcriptional activation functions (AF-2). The F domain is not present on all nuclear receptors, and its function is not clear.

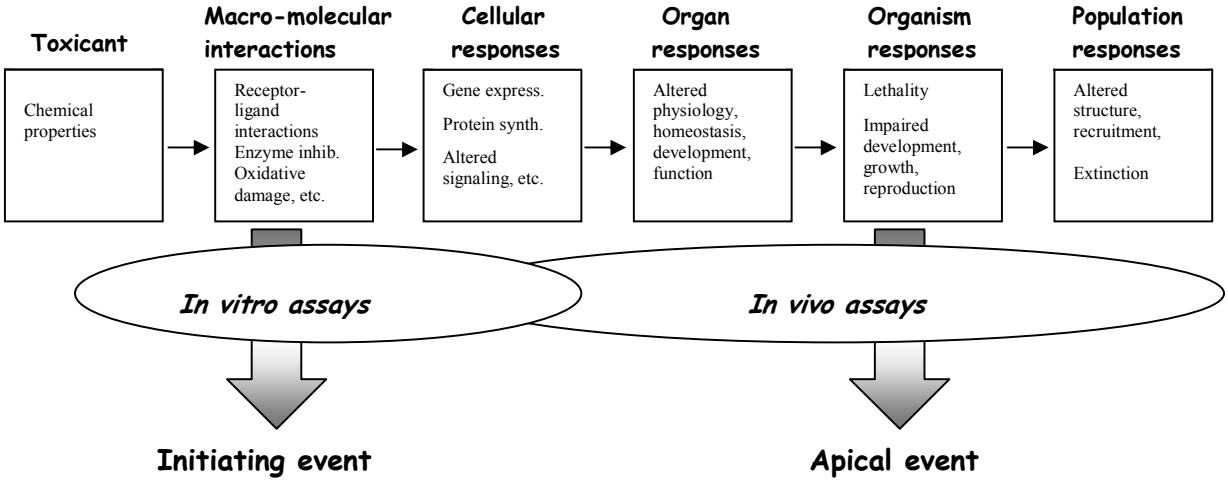

Figure 1-1 Adverse outcome pathway structure depicting the realms of *in vitro* and *in vivo* assays, site of initial interaction with toxicant (initiating event), and site of the typical initial adverse outcome (Adapted from Ankley et al.[4])

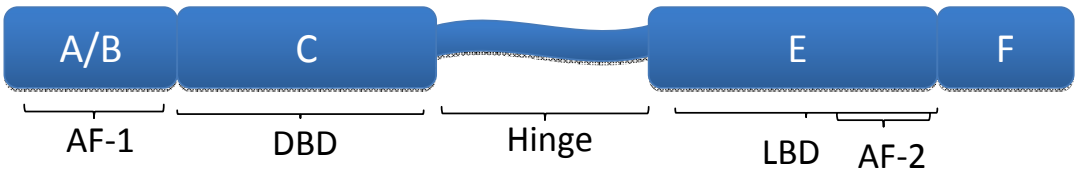

Figure 1-2. Domain structure of hormone nuclear receptors.

7. The susceptibility of peptide hormones, largely of neuroendocrine origin, to the action of EDCs has received relatively little attention. This may be because receptor proteins designed to recognize and bind peptide hormones are less likely to recognize typical environmental chemicals. However, precedent does exist for environmental chemicals modulating the secretion of peptide hormones (e.g., Fraites et al.[6]); therefore, assays for the detection of such disruption will be described in this document. Endocrine signaling pathways for which evidence of endocrine disruption is limited to *in vitro* observations (e.g., MAP kinases) were not included in the DRP. Several endocrine pathways, that contribute to the regulation of apical processes relevant to this DRP (e.g., ghrelin and leptin signaling pathways), are not addressed since no data of endocrine disruption was revealed in our literature search. However, exclusion of such pathways likely indicates the absence of evaluation rather than the absence of disruption by environmental chemicals.

8. The intent of this DRP is to provide methods for both the mechanistic evaluation of the action of EDCs, as advocated in *Toxicity Testing in the 21st Century: A Vision and a Strategy*,[3] and for the assessment of physiological consequences. This document is not all inclusive of neuro-endocrine pathways or the physiological processes regulated by the pathways. Rather, the document covers those neuro-endocrine pathways for which (a) significant evidence of susceptibility to disruption by environmental chemicals with potential for adverse outcome exists; and (b) assay procedures for the detection of environmental endocrine disruption are sufficiently developed for protocol standardization and validation. Chemicals that are known to disrupt each pathway are described in the respective sections. These are not exhaustive lists of known EDCs, but rather are examples of chemicals that may serve as reference compounds in future standardization and validation of the assays. These pathways are diagrammed in **Figure 1-3**.

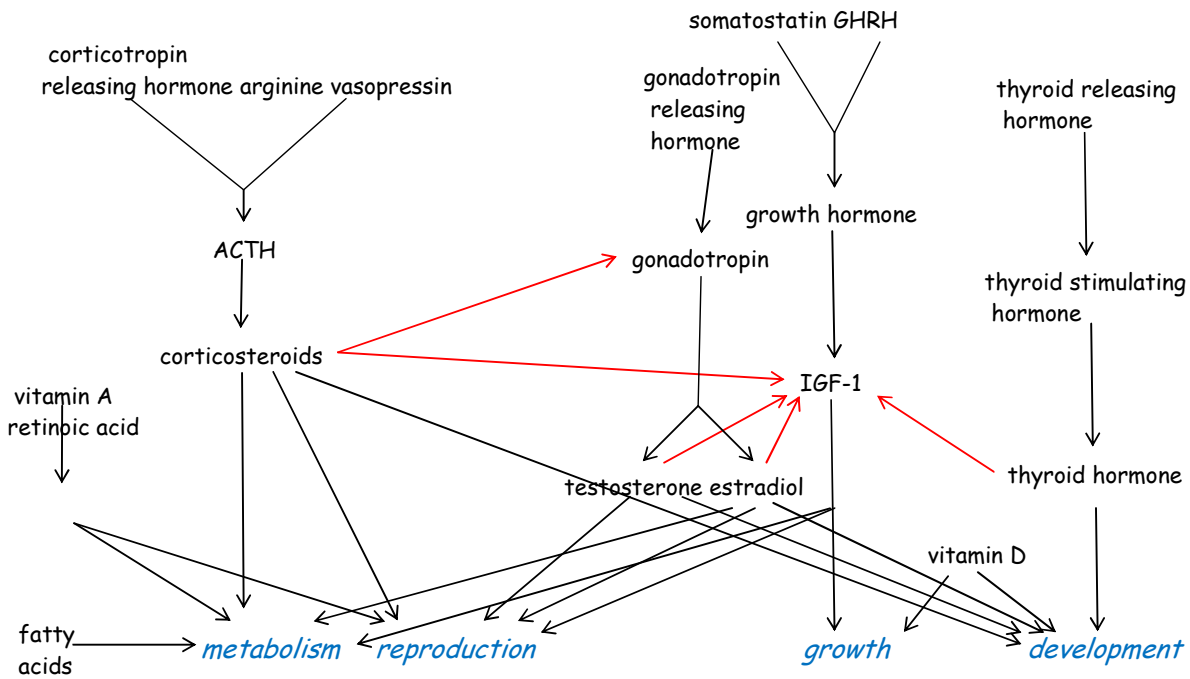

Figure 1-3. Some examples of neuro-endocrine pathways that are affected by EDCs, resulting in symptoms of metabolic syndrome and disruptions in reproduction, growth, and development.

Black arrows denote contiguous pathways. Red arrows highlight examples of cross-talk between pathways.

9. The overall intent of this DRP is to provide guidance on testing approaches that can be used for assessing the actions and toxicity of environmental chemicals on neuro-endocrine pathways not addressed in current Test Guidelines. This DRP is not intended to introduce a new patchwork of disparate tests to add to the existing complement of testing procedures. Whenever possible, approaches for the integration of tests are described so that the greatest amount of information can be derived with the least investment of time, resources, and animals. Effort was made to minimize redundancy among assays; however, the assays are presented in the context of pathways, and pathways are typically branched, rather than linear, with various intersections among different pathways (**Figure 1-3**). Accordingly, some redundancy in assay descriptions was warranted to maintain the integrity of individual pathways. The DRP does not specifically address temporal aspects of susceptibility to EDCs such as effects in old animals exposed in utero (e.g. earlier menopause, reduced testosterone in old males) or hormonally induced cancers (e.g. breast cancer, testicular cancer, prostate cancer), although such considerations are warranted in studies designed to identify "no effect" levels of the chemical. There exist many assays for the clinical evaluation of endocrine function. These assays are typically not addressed in this DRP unless they have been used to assess environmental endocrine disruption. However, such assays do hold promise for incorporation into testing schemes following evaluation for such application.

10. The OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (ENV/JM/MONO(2005)14 describes eight criteria for test method validation. Assays recommended in this DRP were derived from the peer-reviewed research literature and generally do not formally meet criteria such as inter-laboratory reproducibility, extensive use of reference chemicals to determine assay performance, and assay performance under Good Laboratory Practices guidelines. However, assays recommended in the DRP do meet criteria such as existing rationale for the use of the assay, established relationship between the assay endpoint and the relevant biological response, and (reasonably) detailed assay protocols. These criteria are either evident in the descriptions of the assays or in the references provided.

1.1 Relevance of this DRP to Diseases and Syndromes of Contemporary Concern

11. Human populations have experienced increases in various disorders, such as obesity; diabetes; hyperlipidemia; cardiovascular disease; metabolic syndrome; reproductive disorders such as infertility; autism; and attention deficit hyperactivity disorder (ADHD). Many of these disorders have known or suspected environmental contributors, as well as linkages to the endocrine system. Exposure to endocrine disrupting substances has been proposed as possible contributors to their etiology. Examples include the following:

- Obesity, Diabetes, Metabolic Syndrome. Chemicals known as "obesogens" have been shown to alter lipid homeostasis and promote adipogenesis and lipid accumulation.[7] Among the best described obesogens are chemicals that elicit their effect by binding to and activating the PPARγ:RXR receptor complex[8] (**Figure 1-4**). PPARγ:RXR is a positive regulator of adipocyte differentiation and lipid biosynthesis.[9] Perinatal exposure of mice to estrogenic compounds has been shown to result in weight gain at adulthood.[10] Further, stimulation of the glucocorticoid signaling pathway promotes weight gain.[11] The association of weight gain with other disorders, such as type 2 diabetes and metabolic syndrome (which includes hyperlipidemia and cardiovascular disease), has provided added support for a mechanistic linkage between exposure to EDCs and these conditions.
- Testicular Dysgenesis Syndrome. Testicular dysgenesis syndrome describes a set of conditions, including reduced semen quality, undescended testis, hypospadias, and testicular cancer, that are considered to be increasing in incidence in the human population and may have environmental etiology.[12] The hypothesis posits that a cause of this syndrome may be neonatal exposure to estrogenic or anti-androgenic chemicals (**Figure 1-5**). Experiments performed with rodents have shown that neonatal exposure to a variety of chemicals, particularly anti-androgens, do indeed cause abnormalities in male offspring that are consistent with testicular dysgenesis syndrome.[12]
- Autism and Attention Deficit Hyperactivity Disorder. The potential for neonatal exposure to certain chemicals causing disruption in neurodevelopment is well recognized.[13] For example, dioxins have been shown to cause alterations in avian brain development.[14] Epidemiological studies have revealed associations between consumption of persistent organic pollutants via fish by pregnant woman and neurological deficiencies in offspring.[15-17] Brain development is highly regulated by thyroid hormone, and disruptions in thyroid hormone signaling have received the greatest attention as a possible mechanism of neurotoxicity of some environmental chemicals.[17; 18] Associations have not been established between disorders such as autism or ADHD and exposure to EDCs. However, speculation has been raised that increased incidences of such neurological disorders is the consequence of increased neonatal exposure to endocrine disruptors.[13]

1.2 Relevance of this DRP to the Adverse Outcome Pathway approach

In this DRP an initial description is given of the AOP for these pathways, and a first step is taken to try to allocate the methods to the various levels of the CF, this is presented in the tables at the end of each chapter.

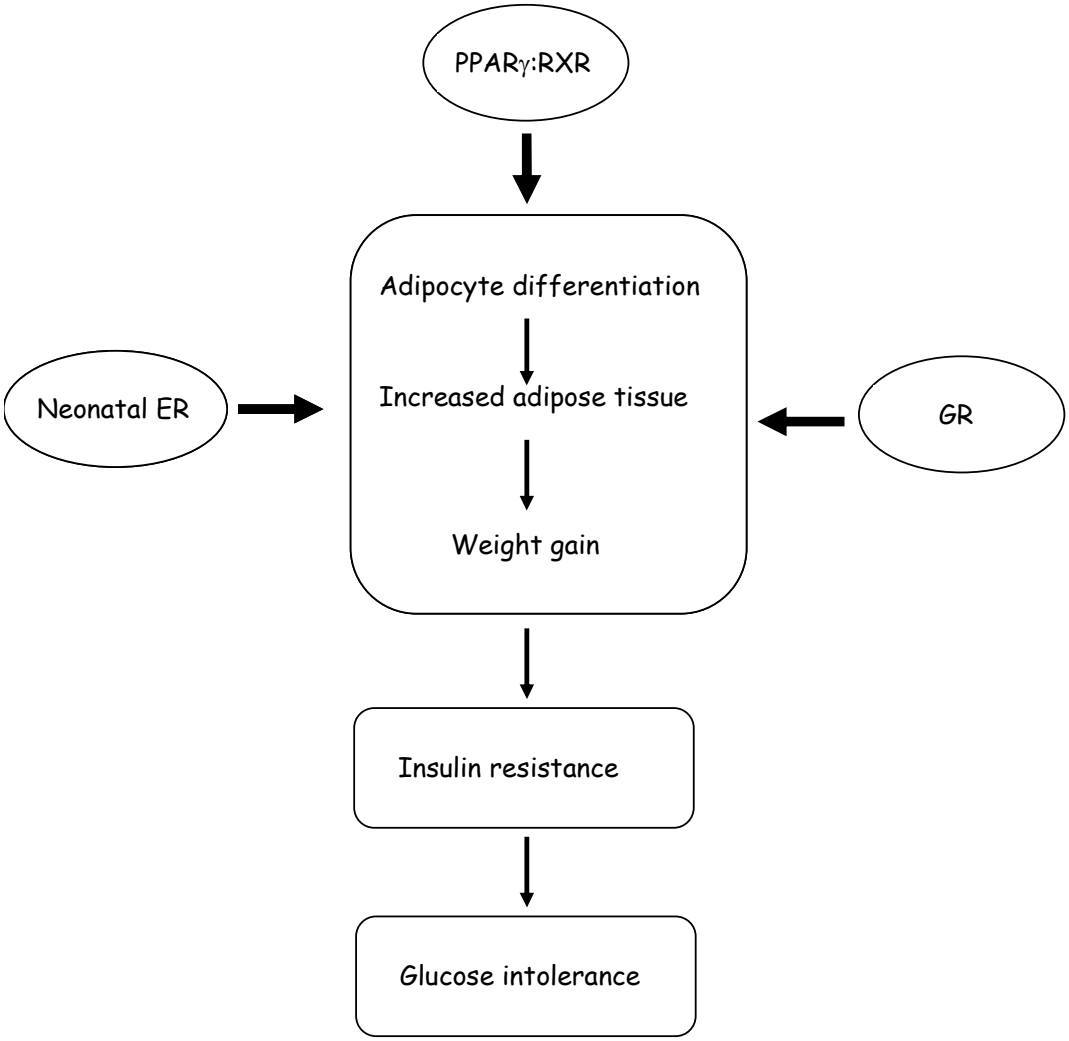

Figure 1-4. Nuclear receptors that stimulate weight gain and associated conditions.

ER, estrogen receptor; PPARγ, peroxisome proliferator activated receptor gamma; RXR, retinoid X receptor; GR, glucocorticoid receptor.

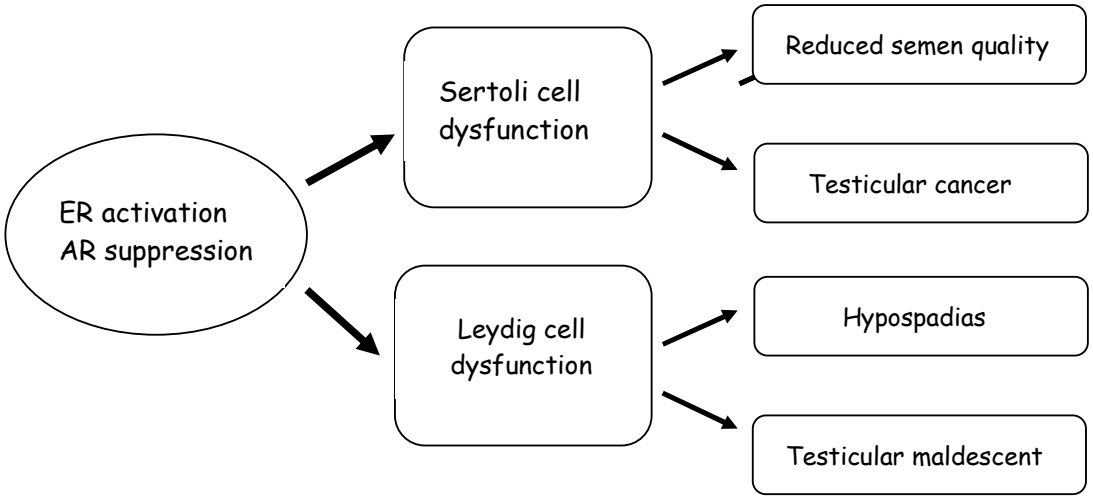

Figure 1-5. Proposed cascade of events leading to testicular dysgenesis syndrome.[12]

THE HYPOTHALAMUS:PITUITARY:ADRENOCORTICAL (HPA) AXIS

2.1 Overview

12. The organization and operation of the vertebrate hypothalamus-pituitary-adrenocortical (HPA) axis (**Figure 2-1**) and its regulation has been the subject of many detailed reviews,[19-22] and only a brief summary is provided here. The HPA axis of vertebrates is primarily a regulator of metabolism,[23] and the HPA axis also has stimulatory and inhibitory effects on the immune system[24] and growth,[19] It also has stimulatory and inhibitory effects on reproduction in vertebrates and is essential for the birth process in at least some mammals.[25; 26] Many aspects of early development, as well as the timing of important events such as puberty and reproductive organ development, are regulated by glucocorticoids from the adrenal cortical tissue in all vertebrate groups.[27] The HPA axis responds to a great variety of stressors and allows the body to respond metabolically to combat the short-term and long-term effects of these stressors. Additionally, the HPA axis affects cardiovascular functions, ionic regulation, and memory. Because of the role of the HPA axis in metabolism, virtually all body tissues are affected by the actions of HPA axis hormones. Numerous human disorders, including obesity and diabetes, are associated with chronic elevation or deficiencies within the HPA axis and are well documented.[28]

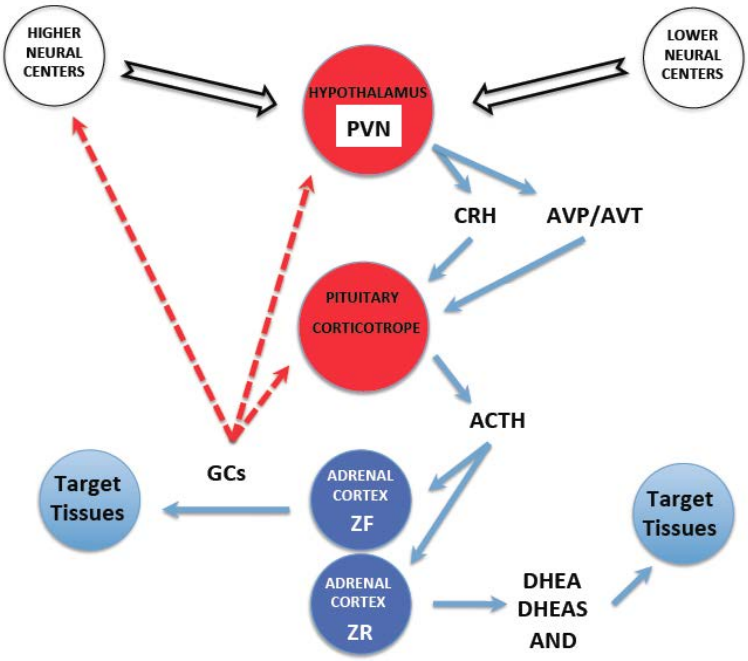

Figure 2.1. The hypothalamus-pituitary-adrenal axis.

PVN, parvocellular nucleus; AVP, arginine vasopressin; AVT, arginine vasotocin; GCs, glucocorticoids; ACTH, corticotropin; ZF, zona fasciculata; ZR, zona reticularis; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; AND, androstenedione.

13. The principal hormones of the mammalian HPA axis are (1) corticotropin-releasing hormone (CRH), produced primarily in the parvocellular neurons of the parvocellular nucleus (PVN) of the hypothalamus; (2) arginine vasopressin (AVP), co-localized with CRH in some PVN neurons; (3) corticotropin (ACTH), produced by corticotropic cells of the pituitary; and (4) the glucocorticoids, i.e., steroids produced in response to ACTH by the cells of the zona fasciculata (ZF) in the adrenal cortex. The principal glucocorticoid of primates and bony fish is cortisol, whereas most other vertebrates, including rodents, secrete primarily corticosterone. Elasmobranch fish produce a unique glucocorticoid, 1α-hydroxy

corticosterone. Additionally, sex steroids (dehydroepiandrosterone [DHEA]; DHEA sulfate [DHEAS]; androstenedione [AND]) are produced by adrenal cells of the mammalian zona reticularis (ZR) following stimulation by ACTH. Fetal adrenals and the placenta also produce estrogens (estradiol and estriol) from adrenal androgens under the influence of CRH.[26] The synthesis and release of glucocorticoids depends upon ACTH and glucocorticoid feedback, primarily at several centers (hippocampus, PVN, pituitary corticotropes), to reduce production of CRH, AVP, ACTH, and adrenal steroids. A variety of neurons originating within the hypothalamus or in other brain regions influence the secretion of CRH and AVP into the hypothalamo-hypophysial portal system (HHPS) and are transported to the pituitary, where they stimulate release of ACTH from the corticotropes. These neurons employ noradrenergic (norepinephrine), dopaminergic (dopamine), serotinergic (serotonin = 5-hydroxtryptamine, 5-HT), and GABAergic (gamma-amino butyric acid), as well as CRH, as neurotransmitters. Numerous additional factors can influence the activity of the HPA axis at various levels, including the urocortins (particularly Ucn I and II), pituitary adenylate cyclase-activating peptide (PACAP), and a variety of interleukins (ILs).

14. The organization and regulation of the HPA axis and the roles of glucocorticoids appear to be very similar in all vertebrate groups, although the typical mammalian zonation is generally absent in fish,[29] amphibians,[30] reptiles,[31] and birds.[32] For example, AVP is replaced by arginine vasotocin (AVT) in non-mammalian vertebrates, although the distributions of AVP and AVT within the brain are very similar (see Moore and Lowry, 1998[33]). However, in fish and amphibians, there is no separate adrenal gland, and the adrenocortical tissue typically is diffusely distributed within the kidneys and often is referred to as interrenal tissue. Here, the HPA axis is used for all vertebrates, although it is frequently called the hypothalamic-pituitary-interrenal (HPI) axis in fish and amphibians.

15. The cells of the zona glomerulosa (ZG) of the mammalian adrenal cortex are responsible for the production of aldosterone, the major mineralocorticoid controlling Na^+/K^+ balance in tetrapod vertebrates. Additionally, excess glucocorticoids also can influence Na+/K+ balance through the mineralocorticoid receptor (GR_1). Secretion of aldosterone is controlled by the renin-angiotensin system and not by the HPA axis, although ACTH maintains the responsiveness of ZG cells to angiotensin II. In bony fish, Na+/K+ balance is regulated in part by cortisol[29] rather than aldosterone. Possible disruption of aldosterone secretion and the consequences for Na+/K+ balance are not considered in this section.

2.1.1 Corticotropin-Releasing Hormone (CRH)

16. Hypothalamic CRH is synthesized in the PVN and is released into the portal circulation connecting the hypothalamus to the pituitary gland. Two CRH receptors have been identified, CRH-R_1 and CRH-R_2. Release of ACTH is mediated through CRH-R_1, located in the cell membrane of the pituitary corticotrope. Occupied CRH-R_1 causes formation of cyclic adenosine monophosphate (cAMP) and activation of phosphokinase A (PKA), resulting in increased availability of calcium ions and release of ACTH. Urocortins also bind to CRH-Rs. Ucn I binds most strongly to CRF-R_1, whereas Ucn II binds more strongly to CRF-R_2. However, Ucn I is not considered to be a physiological releaser of ACTH because it has not been observed in the HHPS. Actions of CRH and urocortins (including Ucn III) in other brain regions also involve these same receptors. Additionally, CRH alters timing of puberty.[34] During pregnancy, placental CRH is instrumental in controlling fetal HPA functions, as well as initiation of birth at least in primates and sheep.[26]

17. CRH also causes release of ACTH from the pituitary corticotropes of non-mammalian vertebrates. CRH and/or CRH-like molecules (e.g., urocortins) have been extracted from the hypothalami of numerous vertebrates.[35] CRH also may have direct behavioral actions as a consequence of its actions in other brain regions of vertebrates.[36; 37]

2.1.2 Arginine Vasopressin (AVP)/Arginine Vasotocin (AVT)

18. In mammals, AVP released from axons of PVN neurons at the median eminence travels via the HHPS to the pituitary and augments the responsiveness of corticotropes to CRH. AVP binds to vasopressin-1 ($V_{1a}R$) receptors in the cell membrane that activate phospholipase C (PLC). In turn, PLC creates inositol trisphosphate (IP_3), which then releases Ca^{2+} necessary for ACTH release from the corticotrope. Parvocellular cells of the mammalian PVN secrete both CRH and AVP.

19. In non-mammalian vertebrates, this role for AVP on ACTH secretion is assumed by AVT,[38-40] where AVT binds to AT2 receptors. In amphibians, AVT receptors also are found on adrenocortical cells of *Xenopus*, and AVT, as well as pituitary adenylate cyclase-activating peptide (PACAP), stimulates synthesis of corticosterone in frogs and salamanders.

20. Magnocellular neurons of the PVN, as well as in the supraoptic nucleus (SON), secrete AVP and send their axons to the pars nervosa of the pituitary gland, from which AVP is released into the general circulation, where it functions as an antidiuretic hormone, causing water retention. Circulating AVP may have cardiovascular pressor effects, especially at higher concentrations. These actions of AVP involve V_{1b} and V_2 receptors and are unrelated to the functioning of the HPA axis. Nevertheless, agents that affect AVP levels in the general circulation might also alter AVP release from the PVN into the HHPS.

2.1.3 Corticotropin (ACTH)

21. Pituitary ACTH is a polypeptide synthesized and released in all vertebrates by pituitary corticotropes under the influence of CRH working through CRH-R_1.[41; 42] In mammals, release of ACTH is enhanced by AVP via binding to V_{1a} receptor and by AVT binding to AT2 receptors in non-mammals. Corticotropes may also produce another AVP receptor, V_3R, which is increased in tumor cells that become very responsive to AVP. ACTH synthesis by the corticotropes is augmented through enhanced cAMP production caused by PACAP produced locally in the pituitary and possibly released from the PVN. The effect of PACAP on increasing cAMP production in corticotropes also is augmented by IL-6 produced locally.[43]

22. In mammals, ACTH binds to melanocortin receptors (MC_2R) on ZF and ZR cells of the adrenal cortex, causing increased cAMP synthesis that, in turn, brings about secretion of glucocorticoids and adrenal androgens (DHEA, DHEAS, aldosterone), respectively. Excessive ACTH secretion, as occurs when glucocorticoids are reduced or cannot be synthesized by the adrenals, can result in increased adrenal androgen production. Adrenal androgens play important roles in puberty, and excess adrenal androgens are associated with fetal and adult clinical disorders. Other vertebrates respond similarly to ACTH.

2.1.4 Luteinizing Hormone (LH) and Chorionic Gonadotropin (CG)

23. Cells of the ZR in the mammalian adrenal cortex that produce adrenal androgens are also responsive to LH from the pituitary, as well as to CG from the placenta. These actions of gonadotropins have not been assessed in non-mammals.

2.1.5 Glucocorticoids

24. Adrenocortical cells are capable of synthesizing glucocorticoids and androgens, primarily from cholesterol via progesterone. Hence, interference with the synthesis of progesterone from cholesterol or with progesterone metabolism, can have repercussions on the ability to synthesize glucocorticoids and sex steroids. Some important enzymes for glucocorticoid synthesis are 3β-hydroxysteroid dehydrogenase (3β-HSD), 11β-hydroxylase (*CYP11B1*), and 21-hydroxylase (*CYP21A1*). In addition, sulfotransferase

2A1 is necessary for production of DHEAS and aromatase (*CYP19*) for estrogens. The steroidogenic acute regulatory protein (StAR) is required to transport cholesterol to the inner mitochondrial membrane, where the first step in progesterone synthesis occurs. Enzymes of the CYP1A family of P450 cytochromes produced in the liver not only metabolize a wide array of drugs and toxic chemicals via activation of the arylhydrocarbon receptor (AhR) but also metabolize adrenal and gonadal steroids. Elevation of these enzymes can reduce circulating levels of adrenal steroids.

2.1.5.1 Glucocorticoid Receptors (GRs)

25. Receptors for glucocorticoids are typically cytoplasmic protein complexes that, when occupied, act as ligand-activated transcription factors that migrate to the nucleus, where they bind to glucocorticoid response elements (GREs) and activate specific genes. Two kinds of GRs have been described, type 1 (GR_1) and type 2 (GR_2). Glucocorticoids bind readily to both GRs, but aldosterone, the principal mineralocorticoid produced by the adrenals of tetrapods, binds only to GR_1, which often is termed the mineralocorticoid receptor (MR), with GR_2 being called simply the glucocorticoid receptor (GR). The receptors in glucocorticoid target cells are typically GR_2, with the exception of the CA-1 neurons of the hypothalamus, where activation of GR_1 is involved in glucocorticoid negative feedback to the HPA axis.

26. Both hyperadrenalism and hypoadrenalism frequently are treated with pharmaceuticals. Synthetic glucocorticoids, such as prednisone, methyl prednisolone, and dexamethasone (DEX), are commonly employed as GR agonists in hypoadrenalism. Similarly, mifepristone (RU486) is frequently employed as a GR antagonist. Metyrapone (metapirone) is a potent inhibitor of the enzyme 11β-hydroxylase and blocks conversion of progesterone to glucocorticoids and may thus enhance production of adrenal sex steroids. Recently, the presence of another glucocorticoid receptor, GRβ, has been documented.[44] However, its physiological role has not been confirmed.

2.1.5.2 CRH and Glucocorticoid-Binding Protein (transcortinprotein and glucocorticoid-binding protein [transcortin])

27. Corticotropin-releasing hormone binding protein (CRH-BP) has been purified from humans and rats and is considered to be an important regulator of the availability of CRH in blood. CRH-BP is an important regulator of plasma CRH in both fetal animals and adults.[26]

28. Transport of glucocorticoids in the blood is the result of binding reversibly to glucocorticoid-binding globulin (CBG, also known as transcortin). CBG ensures higher blood levels of glucocorticoids and reduces their rate of metabolism and excretion. Evidence also suggests that CBG may facilitate transfer of glucocorticoids to their receptors in target tissues. Hence, fluctuations in CBG levels may affect availability of glucocorticoids to target tissues, rates of metabolism, and/or excretion.

2.1.6. Neuroendocrine Regulation of the HPA Axis

29. Secretion of CRH is strongly influenced by a variety of neurotransmitters from other brain regions. For example, extra hypothalamic neurons secreting dopamine from the medial zona incerta of the subthalamus,5-hydroxytryptamine from the raphe nucleus (RN), or NE from the locus caerculeus (LC), stimulate synthesis and release of CRH and AVP by parvocellular PVN neurons via a variety of pathways. GABAergic neurons originating outside of the hypothalamus in the posterior bed nucleus of the stria terminalis (BNST) inhibit CRH and AVP release, whereas CRH-secreting neurons located in the anterior BNST enhance CRH and AVP release.[45]

30. Negative feedback by glucocorticoids occurs through CA-1 neurons (GR_1) of the hippocampus, as well as via CRH neurons of the PVN and in pituitary corticotropes (GR_2). Additionally, input from other

neural centers, including various sections of the limbic system such as the amygdala and the prefrontal cortex, as well as from some lower brain centers, including the BNST, LC, and the RN, can maintain elevated glucocorticoids during prolonged stress in spite of negative feedback by elevated glucocorticoids. CRH synthesis is regulated via a cAMP/PKA pathway that is stimulated by PACAP released from other PVN neurons. Synthesis of AVP in the PVN is also mediated by cAMP and is enhanced by PACAP.

2.2 Consequences of Disruption

31. Perhaps the importance of the HPA axis in maintaining a healthy homeostatic balance explains, in part, why it is so complex, with many factors involved in its regulation. However, its incredible complexity may cause the HPA axis to be susceptible to a wide variety of chemicals at many different levels. EDCs could affect the HPA axis by increasing or decreasing one or more type of neural activity known to alter CRH or AVP/AVT synthesis and release, or they could directly influence responses of the parvocellular neurons in the PVN to various neural agents. The sensitivity of the corticotrope to CRH or AVP/AVT could be altered through changes in CRH or AVP/AVT receptor levels. EDCs could affect receptor levels in the adrenocortical cells or could increase or decrease the activities of the various steroidogenic enzymes in the adrenocortical cells, thus altering steroidal output. Changes in CRH-BP or CGB levels could affect availability of CRH or glucocorticoids, respectively, to target cells and/or influence their rates of metabolism by liver enzymes and ultimate excretion. Additionally, GR receptors in target cells could be affected by EDCs. GR agonists or antagonists not only affect a variety of target cells, but also can influence feedback mechanisms controlling the entire HPA axis. Furthermore, any of the steps in the mechanisms of action by occupied receptors in corticotropes, adrenal cells, or glucocorticoid target cells (e.g., cAMP production, Ca^{2+} availability, IP_3 production) could alter HPA axis functions. Lastly, the close links between the HPA axis and the immune system, thyroid function, metabolism, and growth, as well as development and reproduction, means that there is a potential for any interference of those functions to also affect the HPA axis.

32. From clinical studies of humans,[28] we have learned that alterations in the HPA axis can influence the stress response and osmotic balance. Glucocorticoids also enhance memory recall, but in excess, can cause neurodegeneration and may contribute to dementia. Overstimulation of the HPA system can alter growth and induce obesity, metabolic syndrome, and eventually diabetes mellitus. Excessive glucocorticoid or glucocorticoid-like actions can weaken the immune response system, resulting in increased cancer or other diseases. Excess adrenal androgens can cause masculinization of females and alter fetal development or birth; delay puberty; or completely shut down the reproductive system. Laboratory studies of mammals and other vertebrates indicate similar fates from hyper- or hypoadrenal conditions.

2.3 Precedent Chemicals as Potential Disruptors of the HPA Axis

33. To date, there has been relatively little investigation of EDC actions on the HPA axis of vertebrates. However, the complexity of the HPA axis and its regulation, as well as the many other endocrine pathways with which it interacts, make it a prime target for EDCs in larval and fetal animals, as well as in juveniles and adults.

2.3.1 Steroid Synthesis and Receptor Agonists and Antagonists

34. Natural (e.g., cortisol, corticosterone) and synthetic (e.g., dexamethasone, prednisone) glucocorticoids have been reported in some wastewater effluents[46-49] and in surface waters.[46; 47; 49] Several GR antagonists (e.g., RU486) are also used clinically and might be expected to appear in wastewater effluents. Acute exposure to resveratrol (phytoalexin) or oxybenzone (sunscreen ingredient) enhances basal secretion of

corticosterone in cultured rat adrenal cells,[50] although a separate study reported inhibition of 21-hydroxylase following chronic exposure to resveratrol.[51]

35. Glycyrrhetinic acid, the distinctive compound found in liquorice, stimulates production of adrenal DHEAS through induction of sulfotransferase 2A1.[52] In-vitro studies show that this compound, as well as certain phthalates and organotins, inhibit the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) responsible for conversion of cortisol to cortisone in humans, preventing cortisol from binding to the MR and causing ion imbalances.[53] Observations in rodents indicate a similar role for 11β-HSD2 and hence a potential target for some EDCs.[54]

36. Treatment of hypercortisolism (Cushings disease) may involve treatment with metyrapone, an inhibitor of 11β-hydroxylase or ketoconazole, that also blocks glucocorticoid synthesis.[55] Metyrapone treatment on an experimental basis has also proven useful in blocking emotional memories, such as those that occur with post-traumatic stress disorder.[56] Metyrapone is effective in other vertebrates but is particularly toxic to salmonid fish and should be considered a potential threat should it appear in wastewater effluents.

2.3.2 Metals

37. Long-term exposure to cadmium interferes with the ability of ACTH to stimulate interrenal tissue of rainbow trout (*Oncorhynchus mykiss*)[29; 57] and is linked to chronic stimulation of the hypothalamus and pituitary, as well as to impairment of the stress response, as evidenced in brown trout (*Salmo trutta*), which live their entire lives in cadmium-contaminated streams.[58-60] Other heavy metals may disrupt adrenal function in fish as well.[57; 61] In addition to the adverse effects of cadmium on reproduction in mammals, cadmium also has direct inhibitory effects on corticosterone levels in rats[62] and on guinea pig adrenal cell functions.[63; 64]

38. Organotins (e.g., tributyltin) can prevent the conversion of glucocorticoids to cortisone by inhibiting the enzyme 11β-HSD2. This may result in development of symptoms of excess aldosterone, as well as elevated circulating glucocorticoids.[65]

2.3.3 Neuroactive Chemicals

39. The presence of a variety of neuroactive pharmaceuticals (e.g., fluoxetine; sertraline that can affect 5-hydroxytryptamine receptors and neuroendocrine [5-hydroxytand NE] pathways) and their accumulation in wildlife[66] offers more potential routes for interruption of the HPA axis. Fluoxetine reduces escape behavior at environmentally relevant levels in fathead minnows (*Pimephales promelas*)[67] and reduces aggressive behavior in blue-head wrasse (*Thalassoma bifasciatum*).[68] Levels of corticosterone are also reduced in rats exposed to fluoxetine.[69]

2.3.4 Vasopressin Receptor Agonists and Antagonists

40. Drugs that mimic AVP (e.g., desmopressin and terlipressin) or antagonize AVP actions (e.g., vaptans)[70] are potential EDCs. Several pollutants, such as some PCBs and PBDEs, also interfere with AVP actions in peripheral mammalian systems.[71] These chemicals may also influence the HPA axis of fish and other aquatic animals, although little work has been done in these areas. Neither the natural nor synthetic vasopressins are probably of concern since these peptides would be readily degraded in wastewater systems. However, synthetic vaptans (such as conivapan and lixivaptan) could be a concern, although they have not been reported and perhaps not even examined in wastewater effluents or surface waters.

2.3.5 CRH Receptor Antagonists

41. The $CRHR_1$ antagonists DMP696 and DMP904, developed for treatment of anxiety disorders,[72] may appear in wastewater. These chemicals could pose a threat to the HPA axis of wildlife.

2.3.6 Pesticides

42. Atrazine and the atrazine metabolite desisopropylatrazine elevate circulating ACTH and corticosterone levels in male and female rats.[6; 73] The elevation was not a generalized stress response to stimulation of gastrointestinal afferents, but rather appeared as a targeted effect of the chemical.

43. Endosulfan decreases the responsiveness of dispersed adrenocortical cells from rainbow trout to ACTH.[74] Derivatives of DDT (DDD and DDE, respectively) also reduce the responsiveness of adrenal cells of rainbow trout[75; 76] and tilapia (*Sarotherodon aureus*)[77] to ACTH, as well as reduce the HPA axis response to stress in tilapia[78] and in the arctic char (*Salvelinus alpines*).[79] Most pesticides, however, have not been tested for their ability to affect the HPA axis, but because of observed effects of estradiol and testosterone on the HPA axis, one might expect that a number of pesticides already shown to disrupt reproduction may also affect the HPA axis.

2.3.7 Arylhydrocarbon Receptor (AhR) Agonists

44. Co-planar PCBs, known activators of the AhR, reduce the responsiveness of arctic char (*Salvelinus alpines*),[80] yellow perch (*Perca flavescens*),[57] and tilapia (*Oreochromis mossambicus*)[81] to stress. PCBs and PDBEs interfere with the actions of AVP on ion balance in mammals,[71] although this effect probably does not occur through alterations of the HPA axis, but via stimulation of AhR pathways. PCBs also have been implicated in the secretion of AVP in mammals.[71]

45. Acute treatment with β-naphthoflavone (BNF), another AhR agonist, decreases responsiveness of rainbow trout adrenal cells to ACTH[82;83], as well as the response of liver cells to cortisol.[83] However, more recent studies of acute BNF treatment of rainbow trout show activation of 5-hydroxytryptamine turnover in the hypothalamus[84] and elevation of plasma cortisol and plasma glucose, as well as increased liver glycogenolysis and gluconeogenesis.[85] Acute BNF exposure also decreased the cortisol response to handling stress.[82]

2.3.8 Estrogens and Androgens

46. Compounds in bleached kraft mill effluent (BKME), initially recognized for its androgenic actions on female fish, also cause atrophy of pituitary corticotropes and adrenocortical cells and reduce the normal response to stress in yellow perch.[86] Both estradiol and the weaker estrogenic nonylphenols reduce plasma cortisol levels in the gilthead bream (*Sparus auratus*),[87] and other estrogenic chemicals may also affect the HPA axis. Testosterone also influences hypothalamic synthesis of AVP and CRH.[88]

2.4 In Vitro Assays

2.4.1 Transactivation Reporter Assays

47. Reporter assays that express the human glucocorticoid receptor and a glucocorticoid-responsive reporter gene are commercially available (see **Table 10-1** in Section 10, Summary*, Conclusions, and Recommendations*). These assays can be used to screen chemicals for interaction (agonist or antagonist) with the glucocorticoid receptor. While such assays are promising as a screening tool for glucocorticoid receptor interaction, they have thus far received little attention for such purposes.

2.4.2 Microarrays

48. Expression of genes following exposure of a cell to a glucocorticoid, glucocorticoid antagonist, or glucocorticoid agonist can be monitored by quantifying changes in mRNA levels of specific genes. Standardized microarrays are available from commercial sources for several species that could be used to evaluate changes in glucocorticoid-regulated gene pathways. However, microarrays have not yet been exploited to evaluate EDC impacts on HPA-regulated pathways. Laboratory protocols and validation tests would have to be done for EDC studies.

2.4.3 Cell Culture Systems

2.4.3.1 Corticotropes

49. Corticotropes isolated from mammals[89-91] have been used to study the actions of CRH and/or AVP, as well as the effects of other agents, on synthesis/release of ACTH into the culture medium or on changes in mRNA levels. These *in vitro* systems must be carefully examined since many additional factors can alter the responsiveness of the corticotrope *in vivo* (e.g., cortisol levels, CRH and/or AVP receptor levels, other circulating or local factors such as PACAP).

2.4.3.2 Adrenal Cortical Cells

50. Mouse models of cultured adrenal cells also may be used to develop EDC screening assays, but one is cautioned that mouse adrenal models often differ markedly from results seen in humans.[92] A human adenocarcinoma cell line (H295R) has been developed as a screening assay for chemical factors that interfere with steroidogenesis,[93] using production of testosterone, and estradiol as end products. This system could be validated for measuring glucocorticoids as well. An *in vitro* method for assessing EDC effects on adrenal cells of rainbow trout[94] and *Xenopus*[95] could provide the basis for a simple screening assay specifically directed at fish and amphibians.

2.4.3.3 Glucocorticoid Target Cells

51. Some *in vitro* systems have been described for evaluating metabolic actions of glucocorticoids on mammalian uterine cells[96] liver cells,[97-101] or adipose cells.[102; 103] Additional *in vitro* systems have been explored from fish liver.[104; 105] Such assays could be adapted for assessing chemicals for glucocorticoid agonist/antagonist activity.

2.5 In Vivo Assays

52. *In vivo* assays may be conducted using a variety of vertebrates, but typically, intact fish, rats or mice, and amphibians are employed. It is important to consider the many regulatory factors involved with the HPA axis and the importance of this axis for survival when assessing the effects of EDCs *in vivo*. The HPA axis is both very responsive to change and very resilient in the face of disturbances. Consequently, *in vivo* assessments of EDC interactions should involve measurement of HPA functions occurring in at least two levels.[58] For example, cortisol plasma levels in a downstream population of brown trout exposed to a non-lethal level of cadmium were not statistically different from a reference population of brown trout living upstream of the cadmium source. However, the downstream trout had greater numbers of CRH-positive neurons in the hypothalamus and hypertrophied adrenocortical cells than the reference fish.[60] Subjecting brown trout from these populations to a stress test (e.g., crowding/confinement) showed that the stress response of cadmium-exposed fish was attenuated and required twice the amount of circulating ACTH to reach the same plasma cortisol level by 3 hours.[59] Furthermore, the exposed fish were unable to maintain elevation of ACTH and cortisol beyond 12 hours during the 24-hour stress test,

whereas the unexposed fish could. Measurement of cortisol levels in stressed and unstressed fish would be an adequate *in vivo* bioassay to detect effects of potential EDCs on the HPA axis. Use of a stress paradigm would be a simple way to obtain meaningful information without the more laborious measurements of metabolic or immunological assessments. However, this approach might not detect effects on glucocorticoid target cells. Hence, coupling this approach with a metabolic measurement such as plasma glucose may be desirable.

2.5.1 Mammals

53. Both mouse and rat models have been used to evaluate effects of chemical exposure on the HPA axis.[73; 106; 107] Typically, perturbations in HPA signaling are determined by measuring serum corticosterone and plasma ACTH levels by radioimmunoassay. Using this approach, Fraites et al.[6] demonstrated that atrazine and one of its metabolites activated the HPA in female rats, which may be the explanation for the well-characterized effects of this compound on female rat reproductive function.[108]

2.5.2 Fish

54. Most *in vivo* work has been done to assess HPA axis functions in trout or other larger species. Small species models such as fathead minnows or zebrafish would be more efficient than using larger species,[109] but it would be more difficult to acquire sufficient volumes of plasma to undertake assays of both ACTH and cortisol. Free cortisol is secreted through the gills of fish, can be readily measured in aquarium water, and correlates with plasma cortisol levels (see review by Scott and Ellis, 2007).[110] However, ACTH most likely would not be measurable in aquarium water with any accuracy so that other procedures, such as histology/immunocytology of the hypothalamus, pituitary, or adrenal tissue, might be necessary. Ex vivo approaches also have been used with fish to assess the effect of chemical exposure on the secretion of cortisol by primary adrenalcortical cells *in vitro* following ACTH administration *in vivo*.[111]

2.5.2 Amphibians

55. Since the HPA contributes to the control of metamorphosis in amphibians, *in vivo* assays employing *X. laevis* tadpoles[112] could be utilized for HPA EDC screening. However, one could have difficulty distinguishing between disruption of the mechanism of action of chemicals that affect both the HPA and the HPT axes as reported for endosulfan.[95] Isolated adrenal cells from *X. laevis* and bullfrog (*Rana catesbeiana*) have been used to evaluate direct effects of xenobiotics on corticosteroid secretion in response to ACTH.[95]

2.6 Strengths, Challenges, and Limitations

56. The emphasis of endocrine-disruption studies primarily has been on the HPG axis and secondarily on the HPT axis. Most studies have concentrated on disruption of the actions of reproductive steroids and thyroid hormones, with little focus on higher levels of regulation. Relatively few studies have examined the effects on synthesis of these hormones. Disruption of the HPA axis has been studied mostly in fish, with rather sparse attention paid to other vertebrates. Hence, as yet, there are no established protocols for screening potential HPA disruptors.

57. Testing paradigms must be carefully selected because the test conditions can influence the results following exposures to chemicals. For example, short-term exposures of intact fish to cadmium may increase cortisol release, whereas longer treatments decrease the sensitivity of adrenal cells to ACTH.[57] Similarly, short-term[50] and long-term[51] resveratrol exposure yielded opposite effects. Stress paradigms are very useful ways to examine the integrity of the HPA axis of intact animals following suspected EDC

exposures.[57; 58] However, stressing of fish may yield opposite effects from exposure versus those observed for unstressed fish or for fed versus unfed fish.[80]

58. Many vertebrates exhibit marked circadian[113; 114] and seasonal[115] variations in HPA activity. Typically, peak levels of glucocorticoids parallel reproductive activity. Consequently, attempts to demonstrate inhibitory or stimulatory actions of a suspected EDC may give very different results in wild species at different times of the year.

59. Finally, investigators must recognize the complexity of the HPA axis when devising testing protocols, especially *in vitro*. For example, some disrupting chemicals may not affect GRs and would be missed if only a receptor-binding or DNA array is employed. Hence, initial screening assays need to cover multiple levels within the HPA axis. Furthermore, the interactions demonstrated between HPA, HPG, and HPT axes, as well as HPA involvement in GH secretion, metabolism, and the immune system, indicate that in-vitro assays can never be considered definitive for screening of chemicals for potential HPA axis activity

2.6.1 Stress, the Adverse Outcome Pathway, and Assay Selection

60. Major functions of the HPA axis relate to stress response and maintenance of homeostasis. Accordingly, activation of the HPA axis has limited utility in screening for chemicals that specifically mimic components of the HPA. That is, it is difficult to ascertain whether activation of the HPA following chemical exposure is due to specific chemical-induced disruption or whether the response is a natural physiologic response to the chemical as an invasive stressor.

61. In-silico modeling has suggested that BPA may bind the human glucocorticoid receptor as an agonist.[116] However, we are aware of no studies in which activation of the glucocorticoid receptor by environmental chemicals has been empirically demonstrated. This may be due to high specificity of the receptor for ligand activation or simply the absence of studies designed to evaluate this interaction. Indeed, most demonstrations of environmental endocrine disruption involving the HPA axis have involved suppression of the axis (see examples in Section 2.3, *Precedent Chemicals as Potential Disruptors of the HPA Axis*). Suppression may occur through reduced production of ACTH, reduced responsiveness of adrenocortical cells to ACTH, reduced secretion of corticosteroids, and perhaps, reduced responsiveness of target cells to corticosteroids. Accordingly, Level 1, 2, and 3 assays (OECD Conceptual Framework; **Table 2-1**) should be designed around these endpoints.

62. Reduced stress responsiveness—as determined by lack of responsiveness to physical stress (e.g., confinement), reduced circulating ACTH, and corticosteroid levels—has most often been used to assess whole-organism responsiveness to HPA axis disruption. Nonetheless, chronic suppression of the HPA axis can have detrimental effects on metabolism; hydromineral balance; and the proper function of various organ systems, including immune, cardiovascular, and respiratory.[117] Endpoints relating to these functions ultimately could be incorporated into Level 4 and 5 assays to provide insight into perturbations that may directly infringe upon health and wellbeing of human populations.

Table 2-1. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on corticosteroid signaling pathways*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: GR activation/inhibition; modulation of corticosteroid secretion	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	GR transactivation reporter assay; corticosteroid production by adrenal cells (OECD TG 456)
Organ-level responses Corticosteroid production in response to ACTH or stress; changes in gene expression patterns in exposed cells or organisms	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (Relevant cell-based assays were included in this level)	Corticosteroid analyses animal exposures; microarrays in tissues derived from whole animal exposures (e.g., TG 229, TG 230, TG 231, TG 440, TG 441, GD 140); microarrays using exposed cells
Organ-level responses Altered stress response	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	ACTH and corticosteroid levels, stress responsiveness during prolonged exposures (e.g., TG 206, TG 408, TG 415)
Anchor 2 **Whole organism responses**	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

HYPOTHALAMUS:PITUITARY:GONAD (HPG) AXIS

3.1 Overview

3.1.1 Structure of the HPG Axis

63. In vertebrates, reproduction is primarily controlled by the HPG axis, and the structure of this endocrine pathway is highly conserved in jawed vertebrates (gnathostoma).[118] The hypothalamic neuroendocrine system regulates synthesis and release of the gonadotropins, follicle-stimulating-hormone (FSH), and LH from the pituitary, which in turn stimulate gonadal development, in particular via the induction of sex steroid synthesis. Sex steroids feed back to the hypothalamus and the pituitary, thereby regulating gonadotropin synthesis and release.[119-121] In addition, non-steroidal feedback regulation of gonadotropins by FSH-stimulated gonadal inhibins contributes to the synchronization of the HPG axis at all stages of the life cycle.[121; 122] In lower vertebrates such as fish, the activin/inhibin system plays a role in paracrine regulation of gonadal function,[123] and an autocrine/paracrine activin system in the fish pituitary has been demonstrated.[124] Furthermore, endocrine feedback of gonad-derived activin/inhibin on gonadotropins has been suggested.[125] However, the involvement of inhibins in the regulation of pituitary gonadotropins as true endocrine hormones of gonadal origin, which circulate in the blood stream, has yet to be demonstrated in fish.

64. Among the hypothalamic neuropeptides and neurotransmitters, hypothalamic gonadotropin-releasing hormones (GnRH) are the key factors stimulating gonadotropin release from the pituitary. GnRHs are decapeptides that act via G-protein coupled receptors (gonadotropin-releasing hormone receptors, or GnRH-R). To date, several molecular forms of GnRH and GnRH-R have been identified in vertebrates.[126; 127] In most species, two forms (three in some fish) of GnRH are present: one that is hypophysiotropic, stimulating gonadotropin release from the pituitary, and one that plays a neuromodulatory role in the central nervous system (CNS). The hypothalamus forms an interface between the CNS and the endocrine system, integrating internal (e.g., nutrition, metabolism) and external factors (e.g., temperature, photoperiod, pheromones). Thus, the hypothalamus is triggered by several factors of the CNS and peripheral hormones to maintain physiological homeostasis by regulating pituitary release of tropic hormones, which control the activities of peripheral endocrine glands. Neurotransmitters modulating the activity of GnRH neurons comprise, for example, glutamate, γ-aminobutric acid, noradrenaline, or dopamine.[128-130] It is important to note here that in some fish species, dopamine exerts a potent negative effect on GnRH-stimulated gonadotropin release.[130; 131] In the context of GnRH regulation, the recent discovery of the kisspeptin/GPR54 system revolutionized our understanding of the neuroendocrine regulation of reproduction. In mammals, the kisspeptin/GPR54 system is thought to integrate environmental cues and nutrition to the reproductive axis,[132] and studies in fish and amphibians similarly indicate a key role of Kisspeptides and Kisspeptide receptors (G-protein coupled receptor 54) for gonadotropin secretion and, thus, reproduction in lower vertebrates.[133; 134]

3.1.2 Structure and Actions of HPG Hormones

65. The pituitary gonadotropins are heterodimeric glycoprotein-hormones consisting of a non-covalently linked common glycoprotein-hormone α-subunit (GSUα; also shared with thyroid-stimulating hormone) and a specific β-subunit (FSHβ or LHβ) conferring their biological activity. Once released into the blood stream, the gonadotropins exert their biological activity via G-protein coupled receptors. Except for agnathans (lampreys and hagfishes), which possess only one glycoprotein-hormone,[118; 126] the existence of two gonadotropins (FSH and LH) and their corresponding receptors (FSH-R and LH-R) is well documented in all vertebrates,[118; 135] and both gonadotropins play differential roles in reproduction. In female mammals, FSH action is most important for cyclic recruitment of follicles during the follicular

phase, whereas the LH surge leads to ovulation and the luteal phase.[136] In males, LH regulates androgen-synthesis in Leydig cells, whereas FSH controls Sertoli cell activity, thereby promoting spermatogenesis in conjunction with androgens. In lower vertebrates, particularly in fish, FSH stimulates ovarian development and testicular spermatogenesis during early gametogenesis, whereas LH is predominantly involved in final gamete maturation, leading to ovulation or spermiation.[66; 137]

66. Gonadotropins stimulate gonadal growth and development via the synthesis of sex steroids (i.e., estrogens, androgens, and gestagens) and local growth factors. Generally, in mammals, gametogenesis is regulated by FSH, and steroidogenesis is induced by LH. Estrogen production by the ovary involves LH-stimulated testosterone synthesis in theca cells and subsequent FSH-mediated aromatization to 17β-estradiol in granulosa cells. In the testis, testosterone synthesis in Leydig cells is stimulated by LH, whereas FSH controls Sertoli cell function. In fish, the situation is more complicated because of some degree of cross-activation of the FSH-R by LH and the potent steroidogenic activity of both gonadotropins.[66; 135; 137] The strong steroidogenic activity of FSH in male fish corresponds to the observation that testicular Leydig cells express both the FSH-R and the LH-R, whereas Sertoli cells express only FSH-R.[66] However, species-specific variations from this general pattern have been observed. For example, in zebrafish (*Danio rerio*), FSH-R and LH-R are expressed in Leydig cells, as well as in Sertoli cells.[138]

67. The three classes of sex steroids—estrogens, androgens, and gestagens—are primarily produced by the gonads or other reproductive tissues such as the placenta. Steroidogenesis in the gonads involves the synthesis of pregnenolone from cholesterol and the subsequent conversion to progesterone (P4) and successively to C19 androgens, which can be further aromatized by P450 aromatase (CYP19) to estrogens.[139; 140] In all vertebrates, estradiol is the most common estrogen. On the other hand, some differences exist regarding the presence and role of androgens and gestagens between tetrapods and fish. In tetrapods, testosterone and dihydrotestosterone (DHT) are the principal androgens, whereas in fish, 11-ketotestosterone (11-KT) is considered as the most abundant and potent androgen.[141] Progesterone is the most important gestagen in mammals and also in amphibians; however, in fish, progesterone plasma levels are usually low and other gestagens are predominant. These are, in particular, 17, 20 β-dihydroxypregn-4-en-3-one (17,20β-P) and, in some species, 17, 20 β, 21-trihydroxypregn-4-en-3-one (17,20β-S).[139; 142]

68. The action of sex steroids is classically mediated by nuclear receptors, which act as ligand-dependent transcription factors within the cell nucleus.[143] In mammals, two nuclear estrogen receptors (ERα and ERβ), one androgen receptor (AR), and two forms of progesterone receptors (PR-A and PR-B, which are encoded on the same gene locus) have been identified.[144] Nuclear ER, AR, and PR also have been characterized in amphibians, reptiles, and birds.[145; 146] In most fish, one ERα and two ERβ forms (one ERβ form formerly was named ERγ) are described, e.g., in zebrafish.[147] Furthermore, two AR are found in some fish, such as perciformes, whereas only one AR is found in cyriniformes, including the zebrafish.[148] Two nuclear PR have been described in the African clawed frog (*Xenopus laevis*) and in Japanese eel, whereas in zebrafish, only one PR is present.[149; 150] The distinct types of nuclear sex steroid receptors display differential tissue-specific expression patterns and show peculiarities regarding specificity towards ligands and target gene regulation.[151]

69. Besides the genomic action of sex steroids, the importance of rapid, non-genomic signaling initiated at the cell-membrane is increasingly recognized.[152-154] Receptors involved in rapid estrogen signaling include the membrane-localized forms of ERα and ERβ, and possibly G-protein-coupled receptor 30 (GPR30). Rapid gestagen signaling has been attributed to membrane G-protein-coupled gestagen receptors (mPR) mPRα, mPRβ, and mPRγ and membrane-localized forms of nuclear PR.[152; 155] Furthermore, rapid non-genomic action of androgens is well documented,[152; 153] and a membrane G-protein-coupled androgen receptor has been characterized pharmacologically in fish ovaries.[153]

3.1.3 Function of the HPG Axis

70. The primary function of the HPG axis in vertebrates is to facilitate the production of germ cells and to coordinate reproductive events in relation to body condition and environment. In addition to its function in adult animals, the HPG axis regulates the differentiation of the sex-specific phenotype during early development. In this context, sex steroids play a pivotal role. In females, estradiol is crucially important for reproductive processes, such as differentiation and maintenance of primary sexual characteristics and behavior, proliferation of the endometrium, and for cyclicity of female reproductive events. In oviparous females, estradiol is best known for its role in stimulating the hepatic synthesis of vitellogenin (VTG), a yolk protein.[137] In males, androgens play a pivotal role in the development of the reproductive system and phenotypic sex and are crucial for testicular spermatogenesis/spermiogenesis, as well as for the expression of male sexual behavior.[66; 156; 157] Although estrogens and androgens are generally considered as female or male hormones due to their sex-specific plasma profiles, ER and AR are expressed in many tissues in both sexes, and androgens are converted to estrogens by tissue specifically expressed aromatase (CYP19) in both males and females. In males, estrogens are considered as indispensable hormones for spermatogenesis,[149] and local aromatization of testosterone into estradiol is pivotal for the development of male-specific brain structures.[158] In females, AR knock out revealed that androgens are important for proper ovarian function and mammary development.[159; 160] In female fish, androgens stimulate previtellogenic oocyte growth and seem to be involved in lipid uptake into oocytes during vitellogenesis.[161; 162]

71. In conjunction with estrogens and androgens, gestagens—the third class of gonadal sex steroids—are indispensable reproductive hormones in all vertebrates. In female mammals, progesterone is primarily produced in the *corpus luteum,* and the placenta and its key role in the uterus and mammary gland for initiation and maintenance of pregnancy is well established.[163] Female PR knock-out mice display a variety of reproductive dysfunctions, including impaired ovarian and uterine function, impaired mammary gland development, and absence of sexual behavior.[164] In female fish and amphibians, gestagens, in particular progesterone and 17α,20β-DHP, respectively, are crucially important for final oocyte maturation.[165] In male fish, gestagens induce spermiation[142] and, in all vertebrates including humans, have been shown to facilitate sperm motility via mPRα.[155]

72. Besides their importance for reproduction, sex steroids are pleiotropic hormones modulating many physiological functions, such as metabolism,[166] the immune system,[167; 168] the cardiovascular system,[169] and skeletal homeostasis.[170]

3.2 Consequences of Disruption

3.2.1 (Anti)estrogens

73. The impacts of EDCs depend on the species; sex; the timing/duration, as well as route and dosage of exposure; and the mechanism(s) of action involved. In general, mechanisms of disruption include perturbation of hormone synthesis, transport, and biotransformation. Most attention, however, has been paid to receptor-mediated mechanisms, i.e. mimicking, blocking, or modulation of the interaction of sex steroids with their nuclear receptors. (Anti)estrogenic EDCs are considered in this review as chemicals that interact with estrogen signaling regardless of whether they directly block/activate ERs, or decrease/increase circulating or local estrogen levels. Likewise, the terms (anti)androgenic and (anti)gestagenic are used analogously in the respective sections.

3.2.1.1 Reproduction

74. In lower vertebrates, such as fish and amphibians, most studies on endocrine disruption are related to perturbations of male reproductive physiology due to exposure to estrogenic EDCs, resulting in feminization phenomena such as intersex gonads or shifts in sex ratio.[171; 172] Examples include the occurrence of testicular oocytes and/or an ovarian cavity, as well as unusually high plasma levels of VTG in male roach (*Rutilus rutilus*) from rivers in the United Kingdom.[173] There are also numerous reports with amphibians on the occurrence of intersex and gonadal dysgenesis in the wild.[174] Many observations in the field were corroborated by laboratory studies demonstrating the potency of estrogenic EDC to disrupt normal male sex differentiation and reproduction in fish and amphibians.[172; 175]

75. Although research on disruption of estrogen signaling appears focused on effects in males, EDCs can also interfere with female reproductive function. In fish, there are numerous reports on disruption of female reproductive endpoints in wildlife. Observations include delayed sexual maturity, reduced gonadosomatic indices, increased ovarian atresia, altered levels of sex steroids, and many more.[172; 176] The chemicals and mechanisms of action underlying these effects are often not known, but many findings in the field are corroborated by laboratory studies. Overt estrogen exposure in females can induce ovarian regression via feedback mechanisms exerted on the pituitary gonadotropins.[177; 178] Furthermore, depending on the timing of exposure, antiestrogens (e.g., aromatase inhibitors) have been reported to either lead to female-to-male sex reversal, or to impair female reproduction by reduction of circulating or local estrogen levels.[172; 179] Interestingly, recent studies indicate that in females, biomarkers, such as VTG and levels of testosterone and estradiol, have a good potential to predict fecundity and might be extrapolated to the population level.[180; 181]

76. In humans, much concern regarding EDCs is based upon effects of estrogen exposure on the developing male reproductive system. It was suggested that the increase of reproductive disorders such as cryptorchidism and hypospadias, accompanied by decreasing sperm counts, share a common etiology (testicular dysgenesis syndrome, **Figure 1-5**) and might be a result of exposure to estrogenic EDCs during fetal development.[182] In fact, experimental studies demonstrated that the male mammalian reproductive system is very sensitive to estrogens during fetal development.[183] However, the connection between environmental estrogens and TDS remains controversial, and several other environmental factors, including antiandrogenic EDCs, may contribute to declining sperm quality and TDS in industrialized countries.[182]

77. Males appear to be more sensitive to estrogenic EDCs; nevertheless, overt estrogen signaling can also cause adverse effects in females. One of the best-documented examples of endocrine disruption in humans is the case of diethylstilbestrol (DES), which was used in the 1940–1970s during pregnancy for preventing miscarriages. *In utero* exposure to DES was subsequently linked to rare cases of vaginal cancer and abnormalities of the reproductive tract in women and to numerous adverse effects on the reproductive system in prenatally exposed men.[184] As a consequence, DES has been intensely studied as a model EDC for the effects of developmental exposure to estrogens.[184]

78. Besides direct effects on the developing reproductive system, there is concern about pre- and perinatal EDC exposure, which might result in altered brain sexual differentiation or neuroendocrine reproductive disruption.[185] Importantly, recent studies in rodents and ruminants have shown that estrogenic EDCs altered hypothalamic Kiss1/kisspeptin mRNA levels, with subsequent effects on GnRH and gonadotropin secretion.[186]

3.2.1.2 Metabolism and Growth

79. Several estrogenic EDCs have been reported to impact metabolic pathways and growth. In fish, it was reported that ethinylestradiol (EE2) increased growth of yellow perch (*Perca flavescens*) accompanied by a higher expression of a variety of genes involved in lipid metabolism and growth, including hepatic IGF-1.[187] In tilapia (*Oreochromis niloticus*), on the other hand, EE2 at environmentally relevant concentrations led to reduction of the growth rate, accompanied by decreased IGF-1 and increased VTG expression in the liver.[188] The reason for these species' specific differences in growth response towards estrogens is not known, but it is interesting to note that naturally, yellow perch displays sexual growth dimorphism, with females being bigger than males, whereas in tilapia, the opposite is the case.

80. In mammals, recent research has focused on potential associations between EDCs and metabolic syndrome. Several studies have demonstrated that exposure to environmentally relevant concentrations of EDCs during critical periods of differentiation resulted in obesity (**Figure 1-4**).[10] In humans, BPA exposure in adults has been associated with higher risk of type 2 diabetes[189] and the impact of BPA on insulin synthesis by pancreatic β-cells is equipotent to that of estradiol.[190; 191]

3.2.1.3 Immune System

81. It is well known that sex steroids influence the immune system, and there is good evidence for the involvement of sex steroids in the etiology of several inflammatory pathological conditions.[167] Not surprisingly, EDCs have the potential to modulate immune function, and the mechanisms responsible for these effects have received attention in lower vertebrates[192-194] and in mammals.[195]

3.2.2 (Anti)androgens

3.2.2.1 Reproduction

82. Ecotoxicological studies on endocrine disruption have focused largely on feminization responses due to estrogen exposure in fish and amphibians. However, antiandrogens can lead to related phenotypes. Several laboratory studies with fish provided evidence that antiandrogens can suppress the expression of male secondary sexual characteristics, or impair spermatogenesis and reduce sperm numbers.[196-198] Furthermore, the induction of intersex has been reported in male fish, as well as in amphibians exposed to model antiandrogens,[175; 199-202] suggesting that a shift toward a higher estrogen/androgen ratio may underlie these phenomena. Antiandrogens are also able to suppress the production of the androgen-dependent protein spiggin in male three-spined sticklebacks, which is used as glue for nest building.[203]

83. Compared to fish, a rather limited number of studies have investigated the effects of (anti)androgens in amphibians. Androgen exposure during sexual differentiation of tadpoles leads to masculinization of sex ratio in *X. laevis*, whereas antiandrogens induce feminization.[175] In adult *X. laevis*, the androgen methyldihydrotestosterone induced testicular tissue in the ovary of females, demonstrating the high plasticity of gonads, even after sexual differentiation is accomplished.[204] Although antiandrogens and estrogens can lead to gonadal feminization, both modes of action are not equivalent, though are often difficult to distinguish, as illustrated by the inconsistency of antiandrogens to induce the estrogenic biomarker VTG in male fish.[198] Furthermore, it has been demonstrated that estrogens and antiandrogens induce distinct and differential changes in gene expression patterns in fathead minnow and zebrafish,[205; 206] as well as in amphibians.[199; 202]

84. Although many chemicals present in the aquatic environment are known to act as antiandrogens,[207] their relevance for wildlife is largely unknown. The issue of antiandrogenic EDCs appears underrepresented in the ecotoxicological literature when compared to the huge amount of data related to

estrogenic modes of action. Interestingly, a recent modelling approach[208] provided evidence that feminization/demasculinization of male fish in British rivers is, in part, due to exposure to antiandrogens possibly acting in parallel with estrogenic compounds. In humans, exposure to antiandrogens acting in concert with environmental estrogens is suggested as one factor associated with the increase of TDS in men.[209; 210]

85. In addition to feminization responses, masculinization also has been reported in wildlife vertebrates as a result of overt androgen signaling. The best-documented example is the induction of male secondary sexual characteristics, namely the development of a male-like gonopodium in female mosquitofish (*Gambusia affinis holbrooki*) in the vicinity of a pulp mill in Florida.[211] Since the development of a gonopodium is androgen-dependent, it has been suggested that the observed masculinization was due to exposure to androgenic EDC. In fact, it was demonstrated later on by using binding and AR transactivation assays that the pulp mill effluents exhibited androgenic activity. Another example of an environmental androgen inducing masculinization responses in fathead minnow is the growth promoter trenbolone acetate and its metabolite 17β-trenbolone, which is found in feedlot effluents.[212]

3.2.2.2 Growth

86. Sex steroids, in particular testosterone and its derivates, are anabolic hormones that are known to induce muscle growth in mammals, as well as in fish.[213] Accordingly, interference of EDC with androgen signaling can have effects on metabolism and growth in exposed organism. For example, increased growth was reported for fish exposed to the growth promoter trenbolone or DHT, as well as with methyldihydrotestosterone.[213; 214]

3.2.3 (Anti)gestagens

87. Since gestagens are important regulatory hormones, especially with regard to reproduction, disruption of gestagen signaling can be expected to have significant consequences. However, compared to the (anti)estrogenic and (anti)androgenic modes of action, the possibility that environmental chemicals can alter gestagen signaling has received much less attention. Furthermore, the close interaction of gestagens, androgens, and estrogens with reproductive events poses inherent difficulties attributing any biological effects clearly just to (anti)gestagenic modes of action.

3.2.3.1 Reproduction

88. Disruption of gestagen signaling can have significant adverse effects on a variety of processes relevant for reproduction in all vertebrates. However, since gestagens interact at multiple levels with the signaling of other sex steroids, in particular estrogens, a clear identification of *in vivo* (anti)gestagenic effects might become a difficult task. Furthermore, gestagen action can be mediated by the classic nuclear PR, as well as membrane-bound PRs, and disruption of either pathway may have serious consequences that must be considered in EDC testing.

89. The classical gestagen action in fish and amphibians is induction of final oocyte maturation via a non-genomic pathway.[165] Several pesticides and other environmental chemicals are known to impair fish or amphibian oocyte maturation *in vitro*. For example, Pickford and Morris[215] showed that methoxychlor inhibited progesterone-induced germinal vesicle breakdown (GVBD) in denuded *X. laevis* oocytes. Furthermore, studies demonstrated the inhibition of *in vitro* maturation of fish oocytes by chemicals that also bind to the mPR.[216; 217] Interestingly, stimulatory actions on oocyte maturation also have been reported. For example, Tokumoto et al.[218] showed that DES induced GVBD and cyclin corticosterone synthesis in goldfish oocytes. Recently, Rime et al.[219] demonstrated that the imidazole fungicide prochloraz induced GVBD in intact trout follicles. The stimulatory action of prochloraz was mediated by

an increase of follicular 17,20β-P production, and this effect synergized with LH. Furthermore, gene expression analysis revealed that prochloraz up-regulated the mRNAs of insulin-like growth factors and of steroidogenic enzymes involved in 17,20β-P synthesis. In addition to final oocyte maturation in females, numerous chemicals have been shown to impair sperm motility, probably by binding to mPR on the sperm surface.[220; 221]

90. Recent *in vivo* studies in fish demonstrated severe effects of contraceptive gestagens, sometimes at environmentally relevant concentrations, on gonad development and fecundity in medaka and fathead minnow.[222; 223] Similarly, in amphibians, recent studies suggest strong effects of contraceptive gestagens on the HPG axis,[224] oviduct development,[225] and the thyroid hormone signaling pathway.[226]

91. In mammals, interference with gestagen signaling has been extensively investigated in the context of contraception using synthetic gestagens.[227] However, studies on environmental chemicals disrupting mammalian reproduction with regard to a specific (anti)gestagenic mode of action seem to be rare. Beilmeier et al.[228] showed that pregnancy loss in mammals caused by bromodichloromethane was associated with decreased plasma LH, as well as progesterone levels, and reduced responsiveness of the *corpus luteum* towards LH-stimulated progesterone secretion.[228] Dioxin might also interfere with gestagen signaling since it has been shown to induce endometrial progesterone resistance in mice.[229]

3.2.3.2 Immune System

92. In addition to estrogens and androgens, gestagens also have been reported to be immuno-modulatory hormones. In mammals, modulations of the immune system associated with increased progesterone levels during pregnancy are well documented.[230] Thus, EDCs interfering with gestagen signaling have the potential to affect the immune system in vertebrates. For example, in fish, it has been reported that gestagens inhibited NO release from carp leukocytes.[192]

3.3 Precedent Chemicals

93. Bisphenol A (BPA), phthalate esters, and polychlorinated biphenyls (PCBs) were chosen as example precedent chemicals due to their environmental importance being ubiquitous and the availability of studies dealing with their endocrine disrupting potentials associated with reproductive physiology in humans and wildlife.

3.3.1 (Anti)estrogens

3.3.1.1 Bisphenol A (BPA)

94. BPA is used primarily for manufacturing polycarbonate plastics and epoxy resins and as an additive for plastics.[231] The annual production volume of BPA is around 2.5 million tons,[231] and BPA is ubiquitous in the environment,[232; 233] as well as in human tissue and fluids.[234-236] Based on *in vitro* binding and transactivation studies, BPA is usually considered as a weak estrogen, displaying affinities for nuclear ER being several orders of magnitude lower than that of estradiol.[231] Furthermore, BPA displays antiandrogenicity and antagonistic activity at nuclear thyroid hormone receptors. However, recent studies demonstrated pathways other than binding to classical nuclear ERs, through which BPA can induce cellular responses at very low concentrations.[237] For example, BPA is equally potent as estradiol in activating cellular signal-transduction via membrane ER, namely the membrane-bound form of ERα and GPR30.[236] Some of these responses have been shown to be non-monotonic with regard to dose, and this contributes to the controversies around the human health impact of BPA. BPA also binds with high affinity to the orphan estrogen-related receptor γ (ERRγ),[238] which is highly expressed, particularly in the developing brain.

95. Studies in lower vertebrates have concentrated on classic estrogenic endpoints and have demonstrated feminizing effects of BPA, such as induction of VTG synthesis in male fish.[231] Although these effects were mostly observed at concentrations not reported in the aquatic environment, in some studies, BPA has been shown to feminize sex ratios in amphibians[239] or to disrupt plasma sex steroid levels and to induce changes in gonadal development and gamete quality in fish at environmentally relevant concentrations.[240-242]

96. Concerns about the health implication of BPA in humans is based particularly on so-called organizational effects during exposure at early developmental stages, which can result in irreversible reprogramming of the adult phenotype. In mammals, the prenatal and neonatal periods represent the most vulnerable window of exposure.[183] Studies in rodents reported that exposure to low-doses of BPA during these critical time windows resulted in changes in physiology or organ structure in adults. These effects include altered time of puberty, altered estrous cycles, changes in prostate and the mammary gland, and altered brain sexual dimorphisms.[236] Furthermore, fetal and lactational exposures to BPA have been shown to alter body weight, body composition, and glucose homeostasis in rats. In particular due to rapid signaling via pancreatic ERα, BPA is discussed as a risk factor for type 2 diabetes in humans.[190]

3.3.1.2 Phthalate Esters

97. Phthalates comprise a family of high production volume chemicals, which are used in a variety of consumer products, most frequently as plasticizers in PVC or as additives.[231] Because of their widespread use and the fact that phthalates can leach out of products, they are frequently reported in the environment[243] and in human tissues and fluids.[244; 245] *In vitro* studies show that certain phthalate esters display weak estrogenic[246] or antiandrogenic[241; 247] nuclear receptor-mediated activities. Weak estrogenicity has been confirmed in fish, where phthalate exposure induced VTG synthesis in males and resulted in a low incidence of intersex.[231]

98. In mammals, the ability of phthalates to affect the developing reproductive system in males via antiandrogenic modes of action has been evaluated. In contrast, studies on effects mediated by disruption of estrogen signaling and resulting reproductive effects in females are rather sparse. Exposure of adult female rats to high doses of di-(2-ethylhexyl) phthalate (DEHP) has been reported to result in delayed estrous cycles, reduced plasma estradiol levels, and absence of ovulation, whereas *in utero* exposure to DEHP resulted in delayed puberty in female offspring.[248] Furthermore, Moral et al.[249] reported that *in utero* exposure delayed puberty and induced changes in mammary gland morphology of female offspring. The mechanisms underlying the reproductive effects of phthalates might involve several pathways, including binding to ER, as mentioned above. Interestingly, *in vitro* studies demonstrated that mono-(2-ethylhexyl) phthalate is able to suppress aromatase mRNA and protein levels in rat ovarian granulosa cells, possibly involving PPARs.[250]

3.3.1.3 Polychlorinated Biphenyls (PCBs)

99. PCBs were used in industry as, among others, hydraulic lubricants, dielectric fluids for transformers and capacitors, organic diluents, and sealants.[251] PCBs entered the environment via discharge or accidental release. Although their production was banned, due to their persistence and ability to accumulate in the food chain, PCBs are still found worldwide in the environment and in human and animal tissues.[252; 253] Exposure to PCBs has been associated with a variety of effects, including reproductive, developmental, immunologic, and neurological impairment and carcinogenicity. In general, PCBs are toxicologically differentiated into dioxin-like and non-dioxin-like congeners. Dioxin-like PCBs affect physiology via the AhR, whereas non-dioxin-like PCBs have been shown to exert biological effects via pathways not involving the AhR. Depending on the specific congeners, these PCBs are reported to act as estrogens, antiandrogens, or to change steroid and thyroid hormone levels through mechanisms such as

competing with the natural hormones from their plasma binding globulins or via the modulation of hormone metabolism in the liver.[254; 255] Of special interest is the neurotoxicity of PCBs, which is considered to involve changes in transport mechanisms of neurotransmitters or intracellular pathways, as well as changes in estrogen and thyroid hormone homeostasis and signal transduction.[185; 256]

3.3.2 (Anti)androgens

3.3.2.1 Di-(2-ethylhexyl)phthalate (DEHP)

100. DEHP is one of the most common phthalate esters used as a plasticizer in a variety of consumer products. DEHP acts in mammals as a weak estrogen at the nuclear ER and also displays weak antiandrogenicity via binding to AR. The most important mechanism of action underlying the antiandrogenicity of DEHP, however, seems to be based on distortion of Leydig cell differentiation and migration and reduced testosterone synthesis in the testis, which is accompanied by expression changes in steroidogenic enzymes and of insulin-like hormone 3.[257] In this context, an involvement of PPAR is suggested. However, knock-out studies in mice indicated that the effects of DEHP might be partially independent from PPARα.[248] Interestingly, *in utero* exposure of male rats to phthalates such as DEHP induces several effects also seen in men with TDS, including cryptorchidism, hypospadias, and decreased sperm counts.[258]

3.3.2.2 Flutamide

101. Flutamide is a nonsteroidal antiandrogen that competes with natural androgens for binding to nuclear AR.[259] Therefore, flutamide has been used as a model antiandrogen in a variety of species, including fish,[196; 198] amphibians,[177; 204; 260] and mammals.[209; 261]

3.3.3 (Anti)gestagens

3.3.3.1 Levonorgestrel (LNG)

102. Levonorgestrel (LNG) is a widely used synthetic contraceptive gestagen present in formulations such as the birth control pill, gestagen-only pill, or the emergency contraceptive pill. The contraceptive actions of LNG are based on the prevention of ovulation by exerting negative feedback on pituitary LH secretion and, furthermore, by inducing changes in cervical mucus, suppressing penetrability to spermatozoa. The underlying mechanisms are thought to be mediated via the nuclear PR since LNG displays high affinity to this receptor (323% of the natural ligand).[262] Furthermore, LNG is also androgenic and exhibits affinity to the AR.[262] Although many ecotoxicological studies concentrated on the endocrine-disrupting effects of natural or synthetic estrogens, such as estradiol or EE2, respectively, it is apparent that contraceptive gestagens such as LNG also are present in surface waters at concentrations in the low ng/L range up to 30 ng/L (corresponding to 10^{-10} M).[263-265] Based on a mode of action concept, LNG was considered as a biologically active compound with a high risk to affect non-target organisms in the environment.[266] In fact, a recent study using fathead minnow (*Pimephales promelas*) demonstrated severe suppression of egg-laying at concentrations as low as 0.8 μg/L LNG.[223] Furthermore, it was reported that exposure of *X. tropicalis* to 156 ng/L ($0.5*10^{-9}$ M) LNG during metamorphosis prevented ovarian duct development and impaired oogenesis in females.[225] Exposure to LNG during larval development of *X. laevis* revealed impacts on gonadotropin and sex steroid synthesizing enzyme gene expression and gonadal differentiation of males.[224] These results highlight the diversity of biological actions exerted by synthetic contraceptive gestagens.[227; 262; 267]

103. Mifepristone is also an environmentally relevant antigestagen. However, mifepristone also displays glucocorticoidal and androgenic activities. Thus, *in vivo* effects of this compound cannot solely be attributed to antigestagenic activity with confidence.

3.4 In Vitro Assays

104. Currently, OECD Test Guidelines describe several assays for the detection of classical nuclear receptor-mediated (anti)estrogenic and (anti)androgenic activities of chemicals (e.g., ER binding assay, AR binding assay, ERα transcriptional activation assay [OECD TG 455]). Provided in the respective sub-sections below are complementary approaches that could be used to assess activities not necessarily mediated by the nuclear ER or AR.

3.4.1 (Anti)estrogens

3.4.1.1 ER Transactivation Assays

3.4.1.2 Membrane Receptor Binding

105. Non-genomic signaling pathways of estrogens involve receptors, including the membrane-localized forms of ERα and ERβ, and possibly GPR30.[154] Membrane-associated estrogen receptors can be characterized by simple binding studies of tritiated estradiol to cell-membrane isolations of lower vertebrates, such as amphibians.[175] Characterization of the rapid intracellular signaling pathways mediated by membrane receptor interference involving activation of protein kinases, including ERK1/2 phosphorylation, has been described,[191] but requires further evaluation and validation. ERα and ERβ independent mechanisms exist that trigger estrogenic actions via membrane binding, but screening methods generally involve membrane binding studies[175] and determination of the intracellular signaling pathways by various methods.[268] Species-specific membrane binding experiments in conjunction with ER binding or transactivation reporter assays (e.g., OECD TG 455) would provide a complete molecular assessment of chemical-receptor interactions that may serve as the initiating event in the estrogen adverse outcome pathway (**Table 3-1**).

3.4.1.3 Cell-based Microarrays

106. EDCs that interact with nuclear receptors, such as ERs, induce changes in gene expression of estrogen-sensitive target tissues. Gene expression profiling offers great potential for identifying cellular pathways affected by chemical exposure. Furthermore, the specific expression profile (fingerprint) induced by a chemical of concern can be compared to that of an established reference chemical (e.g., 17 β-estradiol), allowing conclusions on the potential mode of action. Microarrays can be applied to estrogen-sensitive cell lines commonly used for screening of estrogenicity of chemicals. Recently, Terasaka et al.[269] developed a custom array (EstrArray) containing estrogen-dependent genes, characterized the sensitivity and gene expression pattern in MCF-7 human breast cancer cells, and analyzed the compound-specific expression profiles induced by different EDCs (e.g., phyto-oestrogens, phthalates).[269; 270] Assessment of changes in gene expression in cells treated with the chemical being evaluated can provide strong evidence of (anti)estrogenicity. Well-designed assays could provide information on other endocrine activities as well by evaluating changes in various hormone-specific regulated gene pathways (**Table 3-1**).

3.4.2 (Anti)androgens

3.4.2.1 AR Transactivation Assay

107. Assessment of (anti)androgenic EDCs can be performed similarly to (anti)estrogenic ones by AR transactivation assays, demonstrating moderate differences for various EDCs among fathead minnow, rainbow trout, and human AR.[259]

3.4.3 (Anti)gestagens

3.4.3.1 PR Transactivation Assays

108. Several PR transactivation assays have been developed to screen chemicals and environmental samples for (anti)gestagenic activities mediated by the classic PR. These assays are based either on yeast or human cell lines and are usually stably transfected with human PR-A or PR-B. For example, antigestagenic activity by using a recombinant yeast assay was reported for organochlorine pesticides, phenolic compounds, and wastewater treatment plant effluents.[271-273] Antigestagenic activities of polycyclic musks were demonstrated by Schreurs et al.[274] by using the PR Calux assay. A recent study compared a binding assay and two reporter gene assays, the PR Calux and COS-PR, with *in vivo* effects of a variety of chemicals in the McPhail test.[275] The findings from this study showed good correlation between PR binding, transactivational activity in both reporter gene assays, and the *in vivo* gestagenic response.[275]

3.4.3.2 mPR Binding Assays

109. In addition to interactions with nuclear PRs, EDCs are able to bind to mPR and to interfere with rapid gestagen-mediated biological responses. This was shown, for example, by binding studies using membrane preparations from fish ovaries, demonstrating the competitive displacement of the natural maturation-inducing gestagen.[217; 276] Further studies revealed that induction of final maturation of goldfish oocytes by DES was due to binding to mPRα by using membrane preparations of MDA-MB-231 breast carcinoma cells stably transfected with goldfish mPRα.[277] Similarly, binding studies with fish sperm membranes demonstrated the displacement of the natural gestagen by environmental chemicals.[278]

3.4.3.3 Cell-based Microarrays

110. Microarrays have been used to map progesterone-regulated gene pathways in human cells.[279] This approach could similarly be used to evaluate the ability of environmental chemicals to stimulate progesterone-responsive pathways in cultured cells.

3.5 In Vivo Assays

3.5.1 (Anti)estrogens

3.5.1.1 Microarrays

111. Microarray studies have been used to characterize changes in global gene expression patterns of different tissues after exposure to (anti)androgens in fish[280] and in mammals.[281] Furthermore, comparison of effects in fathead minnows induced by estrogens and antiandrogens by real-time PCR revealed clear differences in gene expression profiles in several tissues.[205]

3.5.1.2 Disruption of Brain and Gonad Differentiation

112. It is well known that sex steroids, in particular estrogens, play a pivotal role for brain differentiation during early development and that disruption of these processes can result in persistent changes leading to altered timing of puberty and/or behavioral changes.[185] Recently, studies in zebrafish demonstrated that exposure to very low concentrations of EE2 or nonylphenol during early development resulted in subsequent disruption of forebrain GnRH neurons and aromatase expression in juveniles and adults.[282; 283]

113. Furthermore, it has been shown that the amphibian model *X. laevis* is also very sensitive to aquatic exposure to EDCs with respect to sexual differentiation and gametogenesis, even in adults that possess a high plasticity of gonads within a 4 week exposure to EDCs.[177; 204] The most sensitive parameter investigated was clearly histopathology of gonads demonstrating that EE2 exposure at 10^{-8} M (~3 μg/L) adversely affects in males lobular structure of testis and causes even development of testicular oocytes in males, whereas the antiestrogen tamoxifen at 10^{-8} M affects female gonads by inducing atretic follicles and spermatogenic cysts.[204] In principle, tests could also incorporate further endpoints related to sexual differentiation of the brain. For example, gene expression analysis by qPCR or even visualizing changes in the development of GnRH neurons by immunohistochemistry as demonstrated in zebrafish.[282; 283] Such *in vivo* assays may prove to be diagnostic of estrogenic effects of chemicals involving multiple signaling pathways (e.g., ERα, ERβ, GPR30). However, the standardization and validation of the immunohistochemical methods is warranted because results from these assays can significantly vary among laboratories, and even individual researchers, within laboratories.

114. These apical outcomes would be informative of (anti)estrogenic activity associated with the chemical under evaluation (**Table 3-1**). Current state of knowledge precludes identifying whether these outcomes are mediated by effects on nuclear or membrane receptor signaling; however, such discrimination would have little relevance to risk assessment.

3.5.2 (Anti)androgens

3.5.2.1 Behavioral Changes

115. Changes in behavioral parameters due to exposure to EDCs can be used as a noninvasive and sensitive method to detect disruption of androgen signaling in mammals (sweet preference behavior),[284] fish (nest holding behavior),[285] and amphibians (mate call behavior).[286; 287]

3.5.3 (Anti)gestagens

3.5.3.1 Germinal Vesicle Breakdown (GVBD)

116. Several environmental chemicals have been reported to interfere with final oocyte maturation in fish and amphibians.[215; 216; 288] As a measure for final oocyte maturation, usually GVBD is recorded by visual inspection. Dependent on the use of intact follicles or denuded oocytes, co-incubation protocols with gonadotropin and/or gestagen and the chemical of interest are possible. The assays can be performed either directly *in vitro* or after *in vivo* exposure of the test animals.

3.5.3.2 Sperm Motility

117. The interference of environmental chemicals with sperm motility has been demonstrated in several fish species (e.g., Murack et al.; Thomas and Doughty[220; 221]). Sperm motility can be measured, either after *ex vivo* exposure or after *in vivo* exposure of the test animals. A non-destructive sampling

protocol for obtaining sperm from male fathead minnows was standardized recently, and baseline sperm concentrations and motility were determined.[289] Furthermore, computer-assisted tools for monitoring sperm quality in fish are available.[290]

118. (Anti)gestagenic EDCs should affect biological endpoints in current *in vivo* OECD screening batteries, as has been demonstrated for the fathead minnow[222; 223] and medaka,[222], as well as in rat.[291] In amphibians, the few studies available dealt mainly with larval exposure and suggested that it might be promising to perform additional *in vivo* experiments to assess (anti)gestagenic impacts on adults.[288] However, the diagnostic value of the not yet implemented endpoints concerning potential (anti)gestagenic modes of action appears to be low, and additional investigations (e.g., membrane binding assays and/or PR transactivation assays) are necessary.

119. While the gestagenic signaling pathway is clearly vital to reproduction and has the potential for disruption by environmental chemicals, insufficient information is available to generate a definitive gestagenic adverse outcome pathway (**Table 3-3**). Additional effort is required to establish linkages between molecular initiating events and adverse apical outcomes.

3.6 Strengths, Challenges, and Limitations

3.6.1 (Anti)estrogens

120. Gene expression analyses have great potential to identify mechanisms of action to identify potential biomarkers and to compare responses between animal and human tissues for endocrine disruption.[292] *In vitro* systems offer good reproducibility because effects are measured using the same cellular background. Furthermore, due to the lower biological complexity of *in vitro* systems compared to the situation *in vivo*, data interpretation is more straightforward. This holds especially true when studies concentrate on receptor-mediated pathways and involve a subset of candidate genes. In this context, focused arrays containing a limited number of genes as realized in the above described EstArray might be an appropriate approach to linking molecular initiating events to cellular responses (**Table 3-1**). However, inter-laboratory collaborations are necessary for standardization and validation. Moreover, inter-laboratory reproducibility is necessary for validation. Microarrays also have the potential to evaluate estrogenic responses involving multiple pathways (e.g., ERα, ERβ, GPR30), assuming that cell lines with the signaling capabilities and appropriate positive control chemicals are identified.

121. Current OECD Test Guidelines for screening and testing of endocrine activities of chemicals contain several mammalian and non-mammalian *in vivo* assays. Given the great concern about effects of EDCs on sexual development during sensitive time windows, the need to extend the timing for established test systems seems mandatory. Examples include the fish sexual development test (an extension of the early life stage toxicity test [OECD TG 210]) in which exposure is initiated with fertilized eggs and covers sexual differentiation. For amphibians, an assay also has been suggested that would involve exposure of *X. laevis* or *X. tropicalis* tadpoles during the sensitive stage of sexual differentiation until 75 days post fertilization.[171; 293; 294] In principle, such a "sexual differentiation and metamorphosis assay with *Xenopus*" (SEXDAMAX) would be an extension of the already validated amphibian metamorphosis assay (OECD TG 231) and would cover potential impacts, not only for sexual differentiation but also for thyroid system disruption. Additionally, genetic sex markers have recently been discovered for both *X. laevis* and *X. tropicalis*.[295; 296] Together, this provides an excellent test system to unambiguously demonstrate shifts in the phenotypic sex ratio due to EDC exposure utilizing an amphibian model species. Such modifications of existing Test Guidelines hold promise, but will require additional effort to establish applicability and utility.

122. In order to prioritize potential upcoming methods to assess (anti)estrogenic EDCs, we have to emphasize that estrogenic EDCs have been studied for over two decades. Therefore, the existing *in vitro* and *in vivo* testing methods to determine estrogenic endocrine disruption mediated via nuclear ER interferences are quite well established in mammals, as in lower vertebrates; thus, the development of methods should focus on further modes of action affecting estrogenic signaling, such as membrane-associated effects and antiestrogenic modes of action. The huge knowledge base about estrogen exposure and effects in mammals should focus the interest of research towards ecotoxicological impacts of (anti)estrogens to non-target organisms affected by environmental pollution. Fish and amphibians are well-established models to characterize estrogenic EDCs in non-mammals. However, the complexity of potential endocrine interferences by (anti)estrogenic EDCs cannot become fully covered yet by a combined battery of *in vitro* methods. Thus, there is still a need to utilize *in vivo* assays to provide a holistic assessment of (anti)estrogenic impacts. The gold standards here are full-life-cycle or multigenerational studies. The incorporation of endpoints related to estrogen-regulated aspects of brain and gonad development would expand the application of these assays.

3.6.2 (Anti)androgens

123. (Anti)androgenic EDCs are present in the environment, potentially impacting reproductive health in wildlife and humans.[209] Established *in vivo* assays for the detection of antiandrogenic modes of action include the Hershberger assay using rats (OECD TG 441) or reproduction assays with fish; in particular, the "androgenized female stickleback assay" (variant of OECD TG 230). With regard to the identification of a specific mode of action and the biochemical pathways affected, especially gene expression studies, constitute a promising approach in laboratory studies but also in the field. For example, recent *in situ* studies using caged fathead minnows revealed gene expression patterns in gonad and liver that were characteristic for each of the investigated sites.[280; 285] As more genomic data become available for different species and standardization of experimental design and data evaluation proceeds,[255] it can be assumed that microarrays will become common tools in toxicology.

124. Classical exposure treatments during gonadal development with antiandrogens revealed feminization phenomena in fish and amphibians without differentiating between antiandrogenic and estrogenic compounds. However, using adults of both sexes in parallel seems to be a promising approach to identify androgenic and antiandrogenic modes of action of EDCs and to distinguish antiandrogenic from estrogenic ones because (anti)estrogenic and (anti)androgenic EDCs are characterized by specific patterns of gonad histopathology in male and female adults, as shown for *X. laevis*.[204]

125. One major challenge with regards to ecotoxicological risk assessment of EDCs is to relate changes in biomarkers to population-level impacts. In this context, behavioral tests have a great potential because behavior is an integrative endpoint suggestive for the reproductive success of affected animals. Studies in male sticklebacks showed that both estrogens and antiandrogens can interfere with reproductive behavioral patterns, differentially affecting aggressive behavior towards male conspecifics and courtship behavior, as well as nest building.[297; 298] In amphibians, EDC effects on male reproductive behavior have been demonstrated recently for *X. laevis*.[286] It is interesting to note here that antiandrogen or estrogen treatment induced differential changes in calling parameters following 4-day exposure.[299] In conclusion, reproductive behavior is a valuable non-invasive tool for testing of EDCs, but further research is clearly necessary to associate certain behavioral changes to the specific underlying mechanisms (i.e., estrogenic or antiandrogenic).

3.6.3 (Anti)gestagens

126. Synthetic and natural gestagens are found frequently in the environment;[263; 300; 301] a variety of industrial chemicals or pesticides display (anti)gestagenic activities in PR binding and transactivation

assays.[271; 272] Furthermore, several studies demonstrate severe effects of contraceptive gestagens or other chemicals on gestagen-mediated reproductive processes in fish and amphibians.[221-223; 302] Due to the importance of gestagens for reproduction in all vertebrates, integrating (anti)gestagenic endpoints into existing EDC screening and testing programs seems mandatory. However, the close interaction of gestagens, androgens, and estrogens in regulating reproductive events and the extensive cross talk among these signaling pathways renders attributing specific pathways to outcomes rather difficult. Unambiguous (anti)gestagenic endpoints that could be integrated into *in vivo* Test Guidelines have yet to be identified, though some of the assays described above seem to be promising.

Table 3-1. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on novel estrogen signaling pathways*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: ER-mediated signaling	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	ER transactivation assay (TG 455); embrane binding assay
Tissue-level responses Gene pathway responses in defined tissues or cultured cells	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (Relevant cell-based assays were included in this level)	Microarray analysis using estrogen-responsive tissues derived from *in vivo* exposures (could be applied to any *in vivo* exposure assays); microarray analysis using estrogen-responsive cultured cells
Organ-level responses Disruption of brain or gonad development	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	GnRH neuron development in brain of chronically exposed fish (fish life cycle toxicity test)
Whole organism responses Disruption of brain or gonad development	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Gonad histopathology in chronically exposed amphibians (TG 231) GnRH neuron development in brain of chronically exposed fish (fish life cycle toxicity test)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

Table 3-2. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on novel androgen signaling pathways*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: AR-mediated signaling	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	AR transactivation assay
Tissue-level responses Gene pathway responses	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (Relevant cell-based assays were included in this level)	Microarray analysis using tissues derived from *in vivo* exposures (could be applied to any *in vivo* exposure assays); microarray analysis using androgen-responsive cultured cells
Organ-level responses Behavioral changes	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	Behavioral assessments could be applied to any *in vivo* exposure involving mammals, fish, or amphibians
Whole organism responses Behavioral changes	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Behavioral assessments could be applied to any *in vivo* exposure involving mammals, fish, or amphibians

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

Table 3-3. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on the gestagenic signaling pathway*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: PR-mediated signaling	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	PR transactivation assay; Membrane PR binding assay
Tissue-level responses Progesterone-regulated gene pathway activation	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	Microarray analysis using tissues derived from *in vivo* exposures (could be applied to any *in vivo* exposure assays)
Organ-level responses Germinal vesicle breakdown; sperm motility	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	Assessments in exposed oocytes and sperm *ex vivo* or in oocytes/sperm derived from exposed adults *in vivo* (TG 229, fish life cycle toxicity test)
Whole organism responses Disruption of brain or gonad development	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Reduced fertility in exposed organisms (TG 229, fish life cycle toxicity test)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

THE SOMATOTROPIC AXIS

4.1 Overview

127. The somatotropic axis is responsible for the release of growth hormone and insulin-like growth factor. These hormones regulate a variety of functions related mainly to growth, maturation, and metabolism. The signaling cascade originates at the hypothalamus with the secretion of growth hormone releasing hormone (GHRH) and consists of neuro-endocrine signaling of growth hormone release by the hypothalamic hormones GHRH and somatostatin (also known as somatotropin release inhibiting factor, or SRIF) (**Figure 4-1**). GHRH and somatostatin are released in a coordinate fashion, resulting in a patterned release of growth hormone from the pituitary gland. The secretory patterns of GHRH and somatostatin are influenced by a variety of factors, including sex, age, and circadian timing.

128. GHRH and somatostatin bind to surface receptors of the growth hormone-producing cells (somatotrophs) of the pituitary gland, where they coordinate the pattern of growth hormone release (see Figure 4-1). In rodents and humans, growth hormone secretion occurs in a pulsatile fashion.[303; 304] Adult male secretory patterns are highly regimented with high amplitude, while female secretory patterns are typically less ordered. Sex-specific secretory patterns develop at puberty and are, at least in part, regulated by sex steroids. Studies in rat have demonstrated that the male sex-specific pattern that occurs at puberty is partly programmed in the brain by a neonatal pulse in testosterone production.[305]

129. Growth hormone is delivered via the blood supply to peripheral tissues, where it binds to cell surface receptors that initiate a phosphorylation cascade that involves the JAK/STAT pathway.[306] Elevated growth hormone levels result in insulin resistance, increased blood glucose, and increased lipid metabolism.[307] Tissue responses to growth hormone are dependent upon both the amount of circulating hormone and its pattern of production and release. In the liver, notable effects of growth hormone are in the regulation of CYP enzymes, primarily those involved in steroid metabolism and in the production of insulin-like growth factor-1 (IGF-1) and IGF-2. IGF-1 is the primary cell-signaling form of IGF (see Figure 4-1).

130. IGF-1 is largely responsible for the growth-promoting activities associated with the somatotropic axis, exerting multiple effects at various tissues relating to growth.[308-310] In fish, amphibians, and mammals, IGF-1 and/or IGF-2 contribute to spermatogenesis and/or oocytes maturation.[311-313] Both IGF-1 and IGF-2 also appear to contribute to fetal development in mammals.[314] Serum IGF-1 levels positively correlate to birth weight,[314] and fetal IGF-1 deficiency results in low birth length. IGF-1 also contributes to osmoregulation in fish[315] and to reproductive performance in cattle.[316] A summary of physiological responses to suppression or excitation of the somatotropic axis is presented in **Table 4-1**.

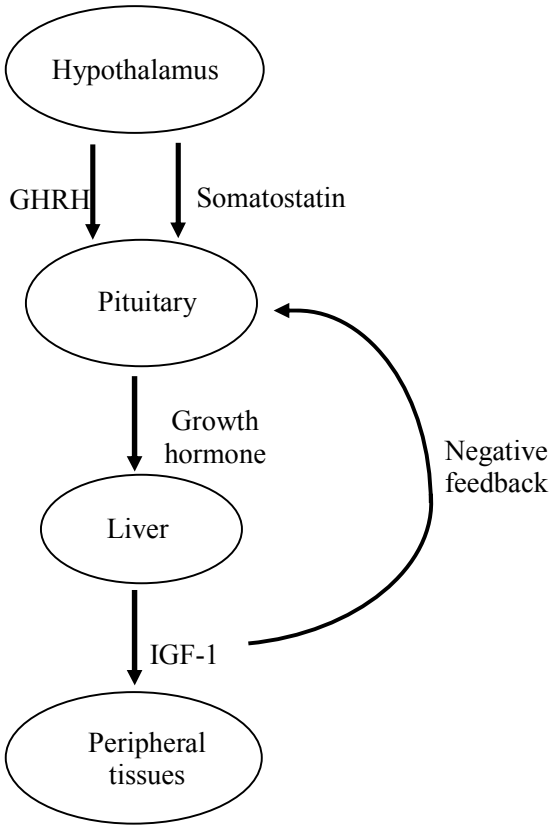

Figure 4-1. The somatotropic axis.

Table 4-1. Some physiological consequences of aberrant suppression and activation of the somatropic axis.

(Summarized from Melmed and Kleinberg[317] for mammals unless indicated otherwise)

Suppression
▪ Increased body fat
▪ Abnormal lipid profile
▪ Impaired cardiac function
▪ Reduced muscle mass
▪ Atherosclerosis
▪ Insulin resistance
▪ Immunodeficiency
Excitation
▪ Increased body size/stature in fish [318]
▪ Heart disease
▪ Thyroid dysfunction in fish [318]
▪ Hypertension
▪ Menstrual disturbances
▪ Sea water tolerance in fish [315]

4.2 Consequences of Disruption

131. Physiological responses to suppression or excitation of the somatotropic axis are known largely through gene knock-out and transgenic overexpression of axis components. The generation of IGF-1 and 2 knock-out mice have clearly demonstrated the respective roles of these hormones in growth. Ablation of the IGF-1 gene resulted in a significant reduction in prenatal and juvenile growth. IGF-1 knock-out mice display delayed bone ossification, muscular dystrophy, and brain abnormalities.[319] IGF-2 knock-out mice have demonstrated the role of this hormone in prenatal growth, but no other deficits have been observed in these animals. Similar effects have been observed in mice in which the growth hormone receptor has been knocked out. These mice exhibit reduced growth, increased body fat, reduced bone mineral density, and reduced mineral content.[320] Transgenic mice that over-produce IGF-1 exhibit increased growth rates resulting in larger animals at adulthood. Transgenic mice that over-express IGF-2 exhibited no overt growth effects.[319] Administration of growth hormone to livestock and the generation of transgenic fish that produce excess growth hormone to enhance somatic growth also have been informative.[321; 322] However, disruption of the somatotropic axis in response to environmental chemicals has received relatively little attention, despite its multi-faceted role in physiology.

4.3 Precedent Chemicals

4.3.1 Estrogenic Chemicals

132. The exposure of fish to estrogenic chemicals has been shown to have a suppressive effect on the somatotropic axis. Exposure of fish to 17β-estradiol, ethinyl estradiol, 4-nonylphenol, genistein, and bisphenol A has been shown to reduce hepatic expression or serum levels of IGF-1, often commensurate with the induction of hepatic vitellogenin synthesis.[323-325] This suppressive effect of estrogens on the somatotropic axis may be mediated by the down regulation of the hepatic growth hormone receptor, preventing the induction of hepatic IGF-1 production by growth hormone.[326-328] This regulatory influence of estrogens on the somatotropic axis has been demonstrated in both mammals and fish. Reduced growth and disrupted smoltification are associated with the exposure of fish to estrogenic chemicals[329] and may be the consequence of the negative regulation of the somatotropic axis by estrogens. Estrogens also can increase IGF-1 levels in specific tissues. For example, estrogen stimulates uterine proliferation in the mouse through the induction of uterine IGF-1 levels.[330]

4.3.2 Anti-thyroid Chemicals

133. Thyroid hormone induction increases somatotropic axis signaling in mammals, birds, and fish.[331-333] Thyroid hormone may stimulate the somatotropic axis through its induction of pituitary growth hormone synthesis[334] or through direct action on hepatic IGF-1 synthesis.[335] Considering the positive regulation of IGF-1 levels by thyroid hormone, it is conceivable that chemicals that suppress thyroid hormone levels may also suppress IGF-1 levels. In addition to eliciting estrogenic activity, BPA has also been shown to bind the thyroid hormone receptor in an antagonistic manner, thus preventing thyroid hormone signaling.[336] This disruption of thyroid hormone signaling may contribute to the suppressive effect of bisphenol A on IGF-1 levels, IGF-1 receptor levels, growth suppression, and altered stress response in juvenile rainbow trout exposed *in ovo*.[337] Similarly, anti-thyroidal PCBs[338; 339] reduced expression of IGF-2 levels in the liver of adult mink (*Mustela vison*).[340] PCB exposure also has been shown to have adverse effects on parameters of growth, including bone development.[341]

4.3.3 Corticosteroid Stimulants

134. Corticosteroids suppress somatotropic axis signaling in fish and mammals.[342; 343] This effect is accompanied by no change in pituitary or plasma content of growth hormone with a decrease in hepatic

IGF-1 gene expression. These observations suggest that corticosteroids desensitize the liver to growth hormone (i.e., suppress expression of the growth hormone receptor) or directly suppress IGF-1 gene expression. Many environmental chemicals have been shown to stimulate corticosteroid production in vertebrates, including some heavy metals, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, and non-chlorinated pesticides (summarized in Di Giulio and Hinton, 2008).[344] As such, exposure to these chemicals would likely suppress IGF-1 levels. Increased plasma corticosteroid levels may sometimes represent a non-specific stress response to the toxicant. However, studies with atrazine have demonstrated that exposure of rats to this chemical elevated cortisol levels without eliciting an overt stress response[6]. Atrazine exposure also elevated cortisol levels in Atlantic salmon (*Salmo salar*) and compromised the ability of smolts to adjust in the transition from fresh to salt water.[345] This effect is consistent with the action of IGF-1 on osmoregulation in fish.

4.3.4 Chemicals that Directly Disrupt the Somatotropic Axis

135. We are aware of no environmental chemicals that interfere, as agonist or antagonists, with growth hormone or IGF interactions with their respective receptors; however, inhibitors of IGF-1 receptor have been designed for possible therapeutic use.[346] As described above, many chemicals can interfere with growth hormone and IGF-1 signaling by interacting with other endocrine signaling pathways that influence the somatotropic axis. These include possible effects on somatostatin, growth hormone, and IGF-1 secretion. The somatotropic axis serves as a central node for many neuroendocrine signaling pathways that are directly susceptible to disruption by environmental chemicals. As such, monitoring of the somatotropic axis can provide a holistic assessment of endocrine disruption in response to chemical exposure. However, this neuroendocrine pathway also is influenced by a variety of environmental signals, including nutrition, season, temperature, and photoperiod.[347] Monitoring of the somatotropic axis may have value in controlled laboratory experiments but may have limited use in field applications.

4.4 In vitro Assays

136. *In vitro* assays described elsewhere in this document for evaluating interactions of chemicals with estrogen, androgen, thyroid, and glucocorticoid signaling would be informative of possible effects on the somatotropic axis as well. Molecular events disrupting these other endocrine pathways may prove to be the initiating event responsible for disruption of the somatropic pathway (**Table 4-2**).

137. Le Gac et al.[348] noted that the *in vitro* incorporation of ^{3}H-thymidine into trout testicular cells increased with increasing exposure to IGF-1. However, co-incubation with prochloraz or nonylphenol ethoxylates both decreased ^{3}H-thymidine incorporation while increasing specific binding of IGF-1 to the cells. The mechanism and significance of this observation are unclear. However, the authors noted that similar effects were observed with Triton® X-100, suggesting that the observed effects may be a consequence of the lipophilic chemicals modifying the membrane characteristics of the cells. At this time, the specificity of this assay is considered to be tenuous, and more research is necessary before this effect and assay could be incorporated into the somatropic adverse outcome pathway.

138. Elango et al.[349] used rainbow trout pituitary explants to evaluate the effects of chemicals on growth hormone secretion. They found that the explants secreted growth hormone over the established timecourse. Exposure to 17β-estradiol or o,p'-DDT significantly increased growth hormone secretion, as did exposure to the anti-estrogens ICI 182 780 and TCDD. The overall stimulatory effect of chemicals, regardless of whether the chemical functioned as an estrogen or an anti-estrogen, raises uncertainties about the utility of this *in vitro* assay. Again, additional research is required before this effect and assay could be incorporated into the somatropic adverse outcome pathway.

4.5 In vivo Assays

139. The reduction in hepatic expression of the growth hormone receptor has been implicated with the suppressive effects of chemicals on the somatotropic axis in mammals and fish. Growth hormone receptor expression can be measured by rt-PCR in a variety of species[350-352] and could be used as an endpoint for somatotropic axis disruption in many of the whole animal OECD Test Guidelines.

140. Analysis of plasma levels or hepatic expression of IGF-1 during *in vivo* assays also would be informative of endocrine disruption via action on the somatotropic axis.[323-325; 337; 342] Hepatic IGF-1 mRNA is typically measured by qPCR, whereas plasma IGF-1 levels are measured by radioimmunoassay. Studies in rodent models suggest that IGF levels may increase in response to light.[353] Analysis of growth hormone levels would be less informative due to the pulsatile nature of growth hormone secretion.[354; 355] The potential for diurnal variations in levels of hormones along the somatotrophic axis necessitates consideration of light regimen during *in vivo* assays.

141. Physiologic studies have shown that IGF-1 levels correlate with fetal birth size in mammals and somatic growth in fish and mammals.[314; 321; 322] While these endpoints have typically not been used to identify chemical disruption of the somatotropic axis, they would likely be informative when evaluating components of a potential adverse outcome pathway (**Table 4-2**).

4.6 Strengths, Challenges, and Limitations

142. Precedent exists for disruptions in the somatotropic axis signaling by environmental chemicals as described above. Consequences of such disruption can be profound, resulting in symptoms associated with metabolic disease and other disorders (see **Table 4-1**). However, we are aware of no demonstration of direct effects of xenobiotics on somatotrope signaling (e.g., growth hormone agonists or antagonists, IGF-1 agonists or antagonists). Rather, the greatest likelihood of effects of xenobiotics on the somatotropic axis is through interactions with endocrine targets that regulate growth hormone and IGF levels (e.g., estrogen, thyroid, corticosteroid signaling). Chemicals shown to target estrogen, thyroid, or corticosteroid signaling in the *in vitro* screening assays should be identified as possible disruptors of the somatropic axis. This disruption could then be confirmed in *in vivo* screening assays (e.g., Level 3 and 4 assays of the Conceptual Framework) or life-cycle studies (e.g., Level 5 assays of the Conceptual Framework) (**Table 4-2**) by evaluating growth hormone receptor or IGF-1 levels in the test organisms as described above. When evaluating IGF-1 protein or mRNA levels, care must be exercised to ensure that unexposed control animals are subject to precisely the same environmental conditions (e.g., handling, photoperiod, sham treatment) since the somatotropic axis is subject to alteration by a variety of conditions in addition to chemical exposure.

Table 4-2. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on the somatotropic axis*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: Estrogen, thyroid hormone, corticosteroid pathway modulation	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	ER (TG 455), TR, and GR transactivation reporter assays
Tissue-level responses Down regulation of the hepatic growth hormone receptor	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	Analyses of hepatic GR mRNA levels in fish and mammalian *in vivo* assays (could be applied to any *in vivo* exposure assays)
Organ-level responses Reduced IGF gene expression	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	Analyses of hepatic IGF-1 mRNA levels in fish and mammals (could be applied to any *in vivo* exposure assays)
Whole organism responses Reduced growth	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Fetal birth weight and length in rodent multigeneration assays (TG 416, TG 443) Growth evaluation in fish assays (fish life cycle toxicity test)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

THE RETINOID SIGNALING PATHWAY

5.1 Overview

143. Vitamin A (retinol) is a fat-soluble vitamin that is derived from dietary sources of both animal and plant origin. Retinol is metabolized to biologically active retinoids (retinoid acids) through oxidative reactions catalyzed by alcohol and retinol dehydrogenases. Retinoid signaling in the body is additionally regulated by the level of retinol and retinoic acid binding to binding proteins and the level of metabolic inactivation largely by members of the CYP26 family of cytochrome P450 enzymes. The retinoid compounds serve as signaling molecules that regulate pleiotropic activities relating to development and differentiation in vertebrates. This hormonal regulatory activity is mediated through association of the retinoids with the RAR (retinoic acid receptor) and the RXR (retinoid X receptor) in vertebrates. Excess or suboptimal levels of retinoids during development result in developmental abnormalities.[356]

5.1.1 Retinoic Acid Receptor Signaling

144. The RAR (NR1B1) is found in vertebrates and chordates, but thus far, has not been identified in protostome invertebrates.[357] Vertebrates typically express three distinct receptors— RARα, RARβ, and RARγ—along with several isoforms of these receptors derived from differential splicing. RARs are best known as receptors for all-*trans* retinoic acid and 9-*cis* retinoic acid, but they also bind and are activated by various metabolites thereof. RAR forms an active transcription factor through its dimerization with the RXR (see below).

145. Excessive RAR-mediated signaling, attained through prenatal, perinatal, and postnatal exposure to exogenous retinoid, results in a variety of development abnormalities.[358-360] These include brachial arch and neural tube defects in mammals;[361; 362] limb malformations in frogs;[363] and fin deformities in fish.[364; 365] Reduced RAR signaling has been shown to cause abnormalities in diaphragm development in rats,[366] abnormalities in blood vessel and bone development in fish,[367] and impaired lens regeneration in frogs.[368]

5.1.2 The Retinoid X Receptor Signaling Network

146. The (RXR NR2B) is an ancient member of the nuclear receptor family and is expressed in lineages ranging from jellyfish (cnidarians) to humans.[369] RXR functions as a master switch in coordinating the activities of multiple components of signaling pathways involved in many processes, including development,[370] reproduction,[371] lipid homeostasis,[372] and metabolism. RXR can self-dimerize forming a homodimeric complex that is activated by ligands such as 9-*cis* retinoic acid and docosahexaenoic acid (DHA). RXR also can form heterodimeric complexes with a variety of nuclear receptors (**Figure 5-1**). Vertebrates typically express three RXR isoforms (α,β,γ).[373; 374] RXR isoforms differ in temporal and tissue-specific expression profiles.[373] Vertebrate RXR heterodimers have been categorized as permissive or nonpermissive.[375] Permissive heterodimers are subject to activation by ligands to either receptor partner. Occupancy of both partners by their cognate ligands can result in synergistic activation of the receptor. Examples of permissive partners to RXR include the PPAR, the liver X receptor (LXR), and the farnesoate X receptor (FXR).[375] Among non-permissive heterodimers, ligand-binding to RXR does not activate the complex. Non-permissive heterodimers are activated exclusively by ligands to the partner receptor (e.g., VDR, thyroid hormone receptor [TR]),[375] the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR) (Baldwin). CAR and PXR serve as activation switches for the biotransformation and elimination of the activating ligands. Activation of RXR by its ligand can result in the synergistic activation of the liganded nonpermissive partner. Noteworthy in this respect is the observed synergistic activation of retinoid signaling when both RXR and RAR are ligand-bound by agonists.[359; 376]

147. Because of its central and obligatory role in the activity of many nuclear receptors, RXR functions in coordinating the regulatory activities of these signaling proteins. The coordinated activities of these receptors serve to achieve the desired physiological outcome. The following are examples of such coordinated activities mediated by RXR.

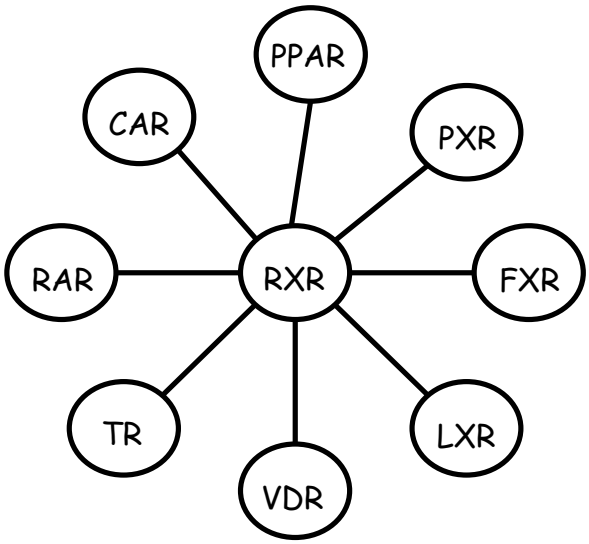

Figure 5-1. Some dimerization partners of RXR that are involved in development, reproduction, and lipid homeostasis.

RXR: retinoid X receptor, PPAR: peroxisome proliferator-activated receptor, Pregnane X receptor, FXR: farnesoid X receptor, LXR: liver X receptor, VDR: vitamin D receptor, TR: thyroid hormone receptor, RAR: retinoic acid receptor, PXR: constitutive androstane receptor.

5.1.2.1 Reproduction in Mammals

148. RXR has multiple roles in regulating male and female fertility and in reproduction. Many of the functions of RXR in regulating reproduction relate to its coordination of the activities of PPAR and RAR. RXR contributes to the development and maturation of both oocytes and spermatids.[377] This activity of RXR appears to be due, in part, to its regulation of steroidogenesis via interaction with PPAR. RXR also regulates aspects of spermatogenesis through its association with RAR. Importantly, the RXR/RAR heterodimer tranduces the retinoic acid signal that determines whether a gamete will develop into a spermatogonium or an oocyte.[378] RXR null mice are infertile.[379] Reduced post-partum signaling of RXR:PPAR in the mammary gland results in the production of toxic milk containing elevated levels of inflammatory lipids resulting in neonatal death.[380; 381] Little is known of the role of RXR in reproduction among non-mammalian vertebrates.

5.1.2.2 Development in Mammals

149. In addition to its significant role in reproductive development, RXR also contributes to other aspects of embryo and fetal development due in part to its association with TR, VDR, and other partner receptors. RXR a has an important role in fetal cardiac morphogenesis and hepatic differentiation.[382] Mice containing an RXRα loss-of-function mutation die as embryos due to gross malformations in the heart. This embryo-lethal phenotype also can be mimicked by vitamin A deficiency. Vitamin A is the precursor to retinoid ligands of RXR. Vitamin A deficiency during fetal development results in impaired brain development with a commensurate loss of expression of RXR and a significant decrease in RAR expression.[383] Similar adverse effects on brain development occur with thyroid hormone deficiency.[384] Together, these requirements for vitamin A and thyroid hormone implicate the RXR:TR heterodimer as a major regulator of fetal brain development. Interestingly, TR knock-mice exhibit developmental deficits in certain aspects of brain development (i.e., neuro-sensory components), but lack the overall disruption in brain development observed in receptor ligand–deficient animals.[385] Clearly, the entire vitamin/hormone signaling network involved in brain development requires further elucidation. Little is known of the role of RXR in non-mammalian vertebrate development. However, considering that the RXR:EcR heterodimer coordinates multiple developmental processes in arthropods,[386] it is likely operative in regulating various aspects of development in non-mammalian vertebrates as well.

5.1.2.3 Lipid Homeostasis in Mammals

150. RXR is a major node in the regulatory network involved in lipid metabolism and homeostasis. RXR forms heterodimeric complexes with several nuclear receptors that are activated by specific lipid ligands. These include PPAR (polyunsaturated fatty acids), LXR (oxysterols), and FXR (bile acids).[372] These receptors typically regulate genes that govern uptake, synthesis, transport, storage, metabolism, and elimination of specific lipid classes.[372; 375] Disruption of the RXR node within this network is associated with metabolic syndrome and associated disorders.[375] The disruption of RXR-mediated lipid homeostasis also has been associated with reproductive and developmental deficits, presumably due to altered availability of lipids that are critical to these processes.[387]

5.2 Consequences of Disruption

151. There are many reports of associations among environmental pollutants, altered retinoid levels in exposed wildlife, and physiological responses consistent with altered retinoid signaling. Fish white sucker (*Catostomus commerconi*) collected from a polluted site had reduced hepatic retinol and retinyl palmitate levels as compared to fish sampled from a reference site.[388] Reduced retinoid stores were accompanied by significant increases in ethoxyresorufin-O-deethylase (EROD) activity and malformations, particularly of the eyes. Flounder (*Platichthys flesus*) that were exposed to polluted harbor sludge experienced reduced hepatic retinoid ester levels in increased CYP1A protein levels.[389] Common terns (*Sterna hirundo*) feeding on fish from polluted areas produced offspring with decreased retinoid ester levels and elevated EROD activity.[390] Affected chicks experienced longer incubation times and reduced body weight at hatching. These examples are highly indicative of exposure to Ah receptor (AhR) agonists.

152. The physiological consequences of activation of RXR by tributyltin have been well described as related to disruptions in lipid homeostasis. In rodent models, tributyltin has been shown to cause differentiation of multipotent stromal stem cells into adipocytes.[7; 391] Stromal stem cells, isolated from white adipose tissue from mice exposed *in utero* to tributyltin, exhibited elevated expression of the PPARγ-regulated gene FABP4. Interestingly, the promoter/enhancer region of the FABP4 gene was hypomethylated in adipose tissue from tributyltin-exposed animals,[391] suggesting that tributyltin-orchestrated epigenetic modifications resulted in changes in lipid homeostasis later in life. Acute exposure of 6-week old mice to tributyltin (0.3 mg/kg body weight) increased the expression of the adipogenic transcript factor C/EBPβ in adipose and testicular tissues.[7] Tributyltin also stimulated increases in the expression of the adipogenic modulators Fatp, Pck1, Acac, and Fasn in liver.[7] This suggests that tributyltin stimulates fatty acid uptake and triglyceride synthesis in the liver. *In utero* exposure of mice to tributyltin also resulted in increase lipid accumulation in adulthood.[7] Similar effects of tributyltin were observed in chronically exposed amphibians (*Xenopus laevis*) and fish (*Oncorhynchus tshawytscha*). Exposed frog tadpoles (1–10 nM aqueous exposure) experienced a dose-dependent increase in ectopic adipocyte formation,[7] while exposed Chinook salmon experienced increased body mass, plasma triacylglycerols, cholesterol, and lipase activity, with increasing tributyltin dose.[392] Taken together, these observations indicated that tributyltin is a high-affinity ligand to the RXR from various species, and exposure results in effects indicative of disruption of normal lipid homeostasis.

5.3 Precedent Chemicals

153. Retinoid signaling has been shown to be disrupted by various, diverse xenobiotics both *in vitro* and *in vivo*. Mechanisms include reductions in endogenous retinoid reserves, retinoid receptor activation by xeno-agonists, and receptor inactivation by xeno-antagonists.

5.3.1 Reductions in Retinoid Levels

154. Aryl hydrocarbon receptor (AhR) ligands such as some polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and PCBs have the ability to disrupt retinoid signaling by depleting endogenous retinoid reserves. The precise mechanism of action resulting in loss of retinoids is not fully understood; however, tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to cause loss of hepatic retinoids,[393] presumably resulting from the mobilization of retinoids from retinyl ester stores,[394; 395] ultimately resulting in the increased renal excretion of polar retinoid derivatives.[394; 396]

5.3.2 RAR Agonists

155. Human RARγ agonists, as measured in yeast two-hybrid assays, include *para*-alkyl-substituted phenolic compounds (4-nonylphenol; 4-*t*-octylphenol; 2-chloro-4-octylphenol; 2,6-dichloro-4-octylphenol; 4-*t*-butylphenol; 2-*t*-butylphenol; 4-*n*-heptylphenol) and styrene dimers (1-phenyltetralin; 1-methyl-3-phenylindan; 1-methyl-1-phenylindan; *trans*-1,2-diphenylcyclobutane; *cis*-1,2-diphenylcyclobutane).[397; 398] RAR β and γ also was activated by the pesticides aldrin, chlordane, dieldrin, endrin, and endosulfan in a transactivation assay.[399] In general, xenobiotics examined are much less potent than endogenous retinoid, with relative potencies ranging from ~0.01 to 1.0% of that observed with all-*trans* retinoic acid.[397]

5.3.3 RXR Agonists/Antagonists

156. Human RXRβ also has been shown to be activated in a two-hybrid assay by various xenobiotics.[400] Among the more potent agonists were 2-tertiary-butylphenol, tetrabromobisphenol, *r*-hexachlorocyclohexane, pentachlorophenol, and 2,4-dichlorophenol. Like RAR agonists, these compounds were relatively weak, with activity observed generally in the concentration range of 10 to 100 μM. Interestingly, some compounds, such as bisphenol A, were inactive in the assay, but with metabolism (an S9 fraction derived from rat treated with methylcholanthrene and phenobarbital was provided in the assay), activation occurred at low micromolar concentrations. Some compounds also were shown to be reasonably competent antagonist of 9-*cis* retinoic acid activity. For example, hexachlorobenzene elicited antagonistic effects at low micromolar concentrations, and hexachlorocyclohexane was antagonistic at high nanomolar concentrations when an S9 fraction was provided in the assay.

157. The most potent RXR agonist identified to date is tributyltin. This compound has been shown to activate RXR (α,β, and γ) in transactivation assays at nanomolar concentrations.[8; 401] *In vitro* transactivation assays also have shown that tributyltin activates some nuclear receptors that function in heterodimeric combination with RXR as lipid sensors. These include PPARγ, PPARδ, LXRα, and NURR1.[7; 402] In contrast to activation of these permissive receptor complexes, non-permissive receptors (e.g., RAR, TR, VDR, PXR) are not activated by tributyltin.[7] These observations provide evidence that tributyltin activates heterodimeric receptor complexes, primarily through interaction with RXR rather than the partner receptor. Triphenyltin oxide has similar potency in activating RXR as tributyltin, while other organotins typically have no (butyltin) or lesser (dibutyltin, tetrabutyltin) activity.[7] RXRs derived from various species, including mammals, amphibian, and even invertebrates, are activated by tributyltin.[7; 403-405] The high potency with which tributyltin activates the RXR stems from its forming covalent bonds within the RXR receptor ligand-binding domain.[406]

5.4 In Vitro Assays

5.4.1 AhR Transactivation Reporter Assay

158. AhR agonists can reduce retinoid stores, resulting in impaired retinoid signaling. *In vitro* reporter assays have been used extensively to evaluate chemicals for their ability to activate the AhR. Early versions of these assays involved measurement of the activity of enzymes induced by the AhR in cultured cells following treatment with the chemical or in liver microsomes from rodents administered the chemical.[407] Typically, the activity associated with the enzyme CYP 1A1 was measured, such as ethoxycoumarin O-deethylase activity. More recently, transcription reporter assays have been constructed and used to detect both AhR agonist and antagonist activity of chemicals. These transcription reporter assays typically have much greater sensitivity than those assays that required induction of endogenous CYP 1A1.[408]

159. Transcription reporter assays consist of a reporter plasmid that contains the gene whose product is easily measured due to its intrinsic fluorescence. This reporter gene is under the control of the dioxin response elements (DREs), which are inserted upstream of the reporter gene transcription start site. This construct is transfected into cells that express the AhR and required co-factors. Cells are exposed to the chemical of interest. If the chemical activates the AhR, then the reporter gene is transcribed and the gene produce is measured using methods appropriate to the assay. Reporter assays have been extensively used in recent years to screen chemicals or chemical mixtures for activity towards the AhR. Many reporters are currently available from commercial sources (e.g., Qiagen, SwitchGear Genomics). Screening services also are provided commercially (INDIGO Biosciences). Since these assays typically utilize AhR that is endogenously produced by the cells used, species' differences in responsiveness can be evaluated using cells from different species. Transcription reporter assays are valued for the sensitivity, low cost, amenability to high-throughput applications, and rapid assay time. Example AhR reporter assays are described in **Table 5-1**.

Table 5-1. Example transcription reporter assays that are used to evaluate activation of the AhR by chemicals.

Species	Cells	Reporter Gene	Source
Human	HepG2 hepatoma	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Human	MCF7 breast tumor	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Human	LS180 intestinal epithelial	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Human	HepG2 hepatoma	Firefly luciferase pLuc1A1	Postlind et al., 1993[410]
Rat	H411e hepatoma	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Guinea pig	GPC16 intestinal adenocarcinoma	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Hamster	AHL lung	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Mouse	H1L1.1c2 hepatoma	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Mouse	MLEL1.1c1 hepatoma	Firefly luciferase pGudLuc1.1	Garrison et al., 1996[409]
Mouse	Hepa 1c1c7 hepatoma	Green fluorescent protein pGreen1.1	Nagy et al., 2002[411]
Rainbow trout	RTH-149 hepatoma	Firefly luciferase pGudLuc1.1	Richter et al., 1997[412]
Zebrafish	COS-1 monkey kidney*	Firefly luciferase pGudLuc6.1	Karchner et al., 2005[413]
Chicken	LMH hepatoma	Firefly luciferase pGL4-ckCYP1A5-6XRE	Lee et al., 2011[414]

Species	Cells	Reporter Gene	Source

*Cells are transfected with the zebrafish AhR and ARNT expression constructs.

5.4.2 RAR Transactivation Reporter Assay

160. Reporter assays have been used for two decades to evaluate retinoid-like activity of chemicals.[415] Early reporter assays utilized chloramphenicol actyltransferase (CAT) as the reporter gene; however, more contemporary assays use reporter genes that code for fluorescent proteins.[416] RAR reporter assays are commercially available (e.g., Invitrogen, Qiagen Company). Commercial screening services using RAR reporter assays are also available (e.g., INDIGO Biosciences). Binding assays have also been used with expressed RAR proteins to assess interactions between receptor and putative ligands.[399; 415] However, these assays are much less informative than are the functional reporter assays because the consequence of binding (receptor activation versus inhibition) cannot be discerned.

5.4.3 RXR Transactivation Reporter Assay

161. Transcription reporter assays have been used to assess both agonistic and antagonistic activity of putative RXR ligands.[400; 417; 418] Commercial kits are available that can be used to screen chemicals for agonist or antagonist activity towards human RXRs (INDIGO Biosciences, Qiagen Company). Reporter assays have revealed that tributyltin is a high-affinity ligand to RXR,[404] the insecticide metabolite methoprene acid,[418; 419] and unidentified metabolites of BPA[400] also activate RXR, but with much lower affinity. Both RAR and RXR reporter assays could serve as a screening assay to discern a potential anchoring molecular event that would trigger assessment along the relevant adverse outcome pathway (**Table 5-2**).

Table 5-2. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on retinoid signaling pathway*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: RXR and RAR activation/inhibition; AhR activation	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	RXR transactivation reporter assay; RAR transactivation reporter assay; AhR transactivation reporter assay;
Organ-level responses Increase retinoid metabolism; reduced retinoid stores; alterations in partner receptor signaling pathways	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	EROD induction; CYP1A mRNA or protein quantification (could potentially be applied to any *in vivo* exposure assay); microarray analyses of relevant signaling pathways in exposed organisms
Organ-level responses	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	
Whole organism responses Excess lipid accumulation	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Weight gain; increased adipose tissue mass, increased lipid accumulation, reduced retinoid levels, microarray analyses (TG 415,TG 416, TG 443, possible amphibian and fish assays)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

5.4.4 Adipocyte Differentiation Assay

162. Experiments performed with the organotin activators of RXR have repeatedly shown that activation, presumably of the RXR-PPARγ receptor complex, causes adipocyte differentiation. Organotins are capable of activating both RXR and PPARγ; however, its much greater potency towards RXR suggests that activation of this permissive complex is due to organotin-binding to the RXR.[7; 403] The ability of chemicals to stimulate adipocyte differentiation can be evaluated in cultured cells. Preadipocyte cells, such as mouse 3T3-L1 or C3H10T1/2 preadipocyte cells, are ideally suited for this assay. Briefly, cultured cells are treated with the putative RXR-ligand, and cells are monitored for several indices of differentiation into adipocytes. A common, simple parameter to measure is the accumulation of oil red O by the cells.[7] Oil red O stains lipids that accumulate in the adipocytes. In addition, triglyceride levels can be measured in cells using commercially available assays.[403] Markers of adipocyte differentiation, such as induction of PPARγ and AP2 mRNA levels,[420] can be measured by real time RT-PCR. This assay is fairly

rapid (<1 week), and endpoints are relatively simple to measure. However, this assay would not likely differentiate between RXR agonists and PPAR agonists. Adipocyte differentiation assays would, however, have value in establishing potential linkages between the relevant anchoring molecular event (RXR activation or PPARγ activation, as discussed in Section 8, *The Peroxisome Proliferator-Activated Receptor Signaling Pathway*) and adverse apical outcomes (**Table 5-2**).

5.4.5 Cell-based Microarrays

163. Microarrays can be used to evaluate changes in the transcription of multiple genes in a manner that would be diagnostic of exposure to RXR agonists or antagonists. Microarrays have been used extensively to evaluate changes in gene expression among cells exposed to RXR agonists.[421-427] However, significant variability in gene responses has been noted, and these differences have beem attributed to cell type used, agonist used, arbitrary selection of threshold response levels, and lack of intra-experiment replication.[428] Analyses of gene expression networks through the use of microarrays hold promise as a holistic tool to assess endocrine disruption via RXR and other pathways. However, standardization of methods is required before the approach can be adopted for routine use.

5.5 In Vivo Assays

5.5.1 CYP 1A1 Induction

164. Measurement of CYP 1A1 mRNA or protein levels, by RT-PCR or immunoblotting respectively, has utility in assessing AhR activity in vertebrate models. Ethoxycoumarin O-deethylase activity also can be measured in hepatic microsomes prepared from exposed animal models. Such approaches incorporate dosage and ADME considerations and can be readily incorporated into existing Test Guidelines (**Table 5-2**).

165. The detection of AhR agonist activity by the above *in vitro* and *in vivo* approaches would signal a molecular event that could lead to decreased retinoid stores and thereby potentially impact both RAR and RXR signaling. This anchoring molecular event may direct testing along an adverse outcome pathway, resulting in retinoid depletion (**Table 5-2**).

5.5.2 Alterations in Retinoid Levels and Metabolism

166. Endogenous retinoid levels can be severely depleted by AhR agonists. *In vivo* analyses of retinoid levels can be measured in animal models. Indeed, analyses of retinoid levels could be incorporated into existing OECD assays involving mammals (uterotrophic assay [OECD TG 440], Hershberger assay [OECD TG 441], two-generation toxicity assay [OECD TG 416]) and fish (reproductive screening assay [OECD TG 229], fish screening assay [OECD TG 230]; androgenized female stickleback screen [AFSS], Medaka multigeneration test);[429; 430] amphibians (*Xenopus* embryo thyroid signaling assay, amphibian metamorphosis assay (OECD TG 231);[431; 432] and avian assays (Avian 2 generation reproductive toxicity assay).[430; 433]Consistent with the relevant adverse outcome pathway, retinoid stores may best be determined following demonstration of the relevant anchoring event (e.g., AhR activation) (**Table 5-2**).

167. Retinoid analyses are typically accomplished by HPLC following liquid extraction of the targeted tissue and separation of polar and apolar derivatives by solid-phase extraction.[434] Typically, exposure to AhR agonists decreases retinoid and retinoid ester levels in the liver and increases levels in the kidney,[435] though variability can exist between species and strain.[436] A promising biomarker of retinoid disruption by AhR ligands is the loss of the retinol metabolite 9-*cis*-4-*oxo*-13,14-dihydroretinoic (DHRA) acid in liver tissue.[437] DHRA levels are significantly depleted following exposure of rats to 0.1 μg/kg TCDD and

are non-detectable following exposure to concentrations >1 µg/kg TCDD.[435; 437] However, the occurrence and behavior of this metabolite in non-rodent species are presently not known.

5.5.3 Alterations in Lipid Levels and Metabolism

168. Changes in lipid levels among mammals used in existing OECD assays and perhaps in other vertebrates could be used as an indicator of endocrine disruption via interactions with RXR. However, maintenance of lipid homeostasis in the whole organism is complex, and changes in lipid metabolism with chemical treatment would not definitively indicate the involvement of RXR. Most notably among lipid-altering effects of RXR agonists on mice and hamster are changes in cholesterol and bile acids. RXR agonists decrease absorption of cholesterol from the intestines and induce mRNA levels of the cholesterol transporter ABC1,which is responsible for the reverse transport of unesterified cholesterol from the inside of intestinal enterocytes into the intestinal lumen.[438] Cholesterol absorption can be measured by providing radio-labeled cholesterol to the test animals and measuring radioactivity in feces as well as in serum,[439] while ABC1 transporter levels can be measured using standard immunoblotting or RT-PCR techniques.[438]

5.6 Strengths, Challenges, and Limitations

169. Considering its obligatory role in several endocrine-signaling processes due to its obligate heterodimerization with other nuclear receptors (see Figure 5-1), RXR signaling should have a prominent role in any endocrine-screening program. Transcription reporter assays have been constructed with RXR from several species, both vertebrate and invertebrate, and this approach should be considered in any *in vitro* battery of screening assays. A similar approach could be adopted for screening of AhR agonist/antagonists, which have the potential to modify retinoid hormone levels (see **Table 5-1**), and RAR agonists/antagonists, which have the potential to disrupt various developmental processes.

170. Microarrays hold promise as a means of assessing the impact of chemical exposure on various endocrine-signaling pathways, including retinoid signaling. However, a comparison of microarray analyses of retinoid signaling revealed a disturbing lack of consistency among assays, as discussed above. Standardization of methods and identification of factors responsible for interassay variability are necessary, before microarrays can be adopted as a screening tool.

171. Adipocyte differentiation assays hold promise as a screening tool, both in cells in culture and in the whole organism. However, endpoints related to adipocyte differentiation may prove to be more holistic general markers of disruptions in lipid homeostasis that may be due to any of a variety of endocrine and non-endocrine processes.

THE HYPOTHALAMUS:PITUITARY:THYROID (HPT) AXIS

6.1 Overview

172. Thyroid hormones are essential for normal physiological functions, including neurodevelopment, growth, and cellular metabolism. Over the course of the past decade, there has been increasing data demonstrating that environmental chemicals disrupt aspects of thyroid signaling and function. These include chemicals that target thyroid hormone receptors as agonists or antagonists, interference with thyrotropin-releasing hormone, altered thyroid hormone synthesis and metabolism, thyroid hormone transport, and others. Chemicals that affect the thyroid hormone systems, either through modulation of the HPT axis or via direct interaction with thyroid hormone nuclear receptors, are termed thyroid disrupting compounds (TDCs). Considering the critical role of thyroid hormones in key physiological processes, it is important to accurately test for potential thyroid toxicants. In 2007, Zoeller et al.[440] reviewed a series of *in vitro* and *in vivo* assays that could adequately capture the range of points within the thyroid endocrine system that may be disrupted by these toxicants across vertebrate taxa. The goal of this document is to provide a current update to the state of recent additions and developments in mechanisms of thyroid disruption and development of novel assays to assess and screen thyroid-disrupting compounds. Here, we provide a brief description of the HPT axis, identify known-thyroid disrupting compounds and their molecular targets within the HPT axis, and present current and promising screening assays to identify putative thyroid-disrupting compounds. For a detailed review and general background information on the HPT axis, the reader is referred to Zoeller et al.[440]

173. Thyroid endocrinology is well conserved across vertebrate taxa. This includes aspects of thyroid hormone synthesis, metabolism, and mechanisms of action.[440] Thyroid hormones are derived from the thyroid gland through regulation of the HPT axis, which is controlled through a complex mechanism of positive and negative feedback regulation. Activation of the HPT is initiated with the synthesis of the tripeptide thyrotropin releasing hormone (TRH). TRH is produced throughout the hypothalamus; however, neurons located within the PVN are the primary site of THR production.[441; 442] Multiple pathways contribute to the synthesis of TRH, including thyroid hormone signaling through feedback mechanisms; leptin and melanocortin signaling; body temperature regulation; and cardiovascular physiology.[443] Each pathway directly targets TRH neurons, which integrate multiple inputs and provide a mechanism to establish set points for TRH production and the thyroid axis at appropriate levels, dependent upon physiological demands. HPT axis signaling is mediated through the paraventricular neurons that project to the median eminence, which is connected to the anterior pituitary gland through hypothalamic-portal vessels.[444] However, in teleosts, the external zone of the median eminence directly innervates the pars distalis of the pituitary.[445] In addition, in frogs and teleosts, a bundle of TRH-containing fibers terminate in the neurointermediate lobe of the pituitary gland, suggesting that TRH exerts multiple, species-dependent hypophysiotropic activities, including stimulation of growth hormone (GH) and prolactin (PRL).[445] Interestingly, in some teleost species and amphibians, TRH does not affect thyroid stimulating hormone (TSH, thyrotropin) secretion. Rather, cortiocotropin releasing hormone acts as a TSH releasing factor.[446]

174. In mammalian systems, TRH is critical for the synthesis and secretion of TSH, either in the presence or absence of thyroid hormones. TSH is a heterodimer consisting of α and β subunits.[447; 448] The α subunit is common to TSH, FSH, LH, and CG. The β subunit is specific to TSH and confers specificity with the TSH receptor. TSH is produced when the anterior pituitary gland receives TRH through the pituitary portal vasculature from the hypothalamus, although paracrine and autocrine activity has been recently described for TRH secreted in the anterior pituitary.[449] TRH signal is mediated through thyrotropin-releasing hormone receptor (TRHR). TRHR is a G protein-coupled receptor in the plasma membrane of the thyrotroph. When bound by TRH, TRHR phosphorylation results in activation of the

phospholipase C second messenger systems, down-stream kinases, and, ultimately, in synthesis and release of TSH from the pituitary.[450] Activation of TRHR by TRH results in denovo synthesis of the TSH beta subunit through defined transcription factors, including cAMP response element-binding protein (CREB)-binding protein and pituitary-specific transcription factor Pit-1.[451-453] In addition, TRH stimulates post-translational glycosylation of TSH, which is critical for TSH heterodimerization, secretion, and bioactivity of mature TSH.[454; 455]

175. TSH released from the anterior pituitary binds to receptors on the cell surface of thyroid follicle cells.[456] TSH receptors are also G protein-coupled receptors, and when activated, stimulate the adenylate cyclase and the cAMP secondary messenger kinase cascade. This includes phosphorylation of PKA and subsequent phosphorylation of transcription factors such as cAMP-responsive element modulator (CREM) and CREB.[457] There is some evidence that TSH additionally activates protein kinase C (PKC) and diacylglycerol signaling pathways.[458] The effects of receptor activation are multifunctional, including increased uptake of iodide into the thyroid cells, iodination of tyrosyl residues on thyrogloubin (TG), synthesis and oxidation of TG, TG uptake from thyroid colloid, and production of thyroid hormones T4 and T3.[440]

176. Iodine uptake in the thyroid gland is governed through the actions of the sodium-iodide symporter (NIS).[459; 460] NIS is located on the outer plasma membrane of the thyrocyte and couples inward-intracellular transport of iodine with sodium ions (Na^+). A Na^+ gradient is established through activity of the Na+/K+ -ATPase and concentrates Na^+ ions three to five times greater on the outside of the cell. Through this process the thyroid gland can concentrate iodine 20 to 40 fold. NIS gene transcription is under regulatory control of TTF1, TTF2, and Pax8, which are activated by PKA, which in turn is stimulated by TSH.[461] NIS is also auto-regulated, where excess iodine accumulation suppresses NIS gene expression.[462] Once iodine molecules are transported into the cell, they are bound to tyrosine residues of thyroglobulin protein as either mono-iodothyronine or di-iodothyronine. As with NIS, thyroglobulin is under regulatory control of TTF1, TTF2, and Pax8 within the thyrocyte and, thus, de novo synthesis of thyroglobulin production is stimulated by TSH.[463-465] Thyroid hormones T4 and T3 are produced through a series of peroxidation reactions that require iodide, hydrogen peroxide, the enzyme thyroperoxidase, and the iodine acceptor protein thyroglobulin.[465] Hydrogen peroxidase is produced through the activity of DUOX/ThOX oxidase enzymes located at the apical pole of the thyroid follicular cells.[466; 467] Thyroid peroxidase (TPO) facilitates covalent attachment of iodide by reducing H_2O_2 and oxidizing iodine where they bind to distinct tyrosyl residues on the thyroglobulin protein forming digoxigenin or mono-iodothyronine.[466; 467] Two digoxigenin molecules form T4, and one digoxigenin and mono-iodothyronine molecule form T3.

177. TSH additionally stimulates secretion of thyroid hormones (T4 and T3) stored in the colloid via endocytosis into the central circulation. This process is mediated through activation of the TSH receptor, intracellular accumulation of cAMP, and subsequent transport, regulation, and proteolysis of TG, resulting in liberation of T4 and T3.[440] Once in the blood stream, thyroid hormones are either bound to transport proteins, thyroid binding globulin, transthyretin, or albumin, or circulate freely in the plasma. The fraction of free T4 and T3 is small (~0.5% of total serum hormone) relative to bound forms. In humans, 75% of serum T4 is bound to TBG, 15% to TTR, and <5% to albumin.[468] While TBG is the predominant thyroid hormone-binding plasma protein in humans, this protein is lacking in adult rats. TBG has much greater binding affinity towards thyroid hormone than do the other two thyroxine-binding proteins. The lack of TBG in the adult rat is an important difference in thyroid hormone physiology between humans and rats and likely contributes to sensitivity differences to some TDCs. Thyroid-binding proteins play an important role in regulating circulating levels of thyroid hormone concentration and represent a large extrathyroidal pool of T4 and T3. Binding of T4 and T3 to these macromolecules serves as a mechanism to regulate spatial and temporal transport of thyroid hormone to target sites and may also provide a mechanism to control iodine clearance.[469] Thyroid hormone levels are also controlled by three

distinct deiodinases, enzymes that are responsible for the conversion, recycling, and degradation of T4 and T3. Deiodinases exhibit specific temporal and spatial expression differences and are responsible for local synthesis of T4 and T3 within the thyroid, the peripheral and local conversion of T4 to T3 (the biologically active form of TH), breakdown of reduced T3 (rT3), and inactivation of T3.[470; 471] In addition to deiodination, thyroid hormones are metabolized in the liver and kidney through conjugation with sulfate or glucuronic acid.[470; 471]

178. At the site of action, bioactive T3 either diffuses passively across the cellular membrane or is actively transported into the cell. TH hormones are lipophilic and were originally thought to enter the cell solely via passive diffusion. More recently, however, there is evidence that THs undergo facilitated and/or active transport across the plasma membrane. Several stereoselective T4 and T3 transporters have been identified, including organic ion transport proteins (OATP) and members of the monocarboxylate transporter (MCT) family.[472-474] Once within the cell, thyroid hormone signaling is mediated through hormone ligand interaction with TRs. TRs are members of the nuclear hormone receptor superfamily. These receptors are ligand-dependent transcription factors that are governed through ligand-dependent interactions, DNA-dependent interactions, and co-regulator-dependent interactions. Multiple forms of the thyroid receptor (THα, THβ1, and THβ2) facilitate transcriptional activation and repression of target genes through interaction with thyroid hormone response elements within the promoter/enhancer region of each gene.[473; 475] T3 binds to each of the TRs with near equal affinity and exhibits an approximately 50-fold greater affinity for TRs than does T4.[476] However, there is some evidence of selective functional activation of T3 with each receptor that may be co-regulator–dependent.[440] TRs also exhibit significant temporal and tissue-specific expression patterns, providing a mechanism to enhance selectivity of thyroid hormone response(s). There are numerous genes that are affected by transcriptional activation of TRs, each highly cell specific. In the case of negative feedback to the hypothalamus and pituitary, T3 binding to the THβ receptor results in ligand-dependent repression of gene transcription and subsequent reductions in THR and TSH levels. Additional nuclear receptors, including RXR, the TR receptor obligate heterodimerization partner, and PPARγ, also function to regulate *Trh* gene expression within the hypothalamus.[477; 478] Conversely, in peripheral tissues, TH results in TR ligand–dependent activation of genes associated with development, growth, and metabolic control.[440]

6.2 Consequences of Disruption

179. Exposure to a wide range of structurally diverse environmental chemicals, including PCBs, dioxins (tetrachlorodibenzo-p-dioxin, TCDD), polychlorinated dibenzofurans (PCDFs), bisphenol A (4,4' isopropylidenediphenol or BPA), polybrominated diphenyl ethers (commonly known as flame retardants), phthalates, perchlorate; halogenated pesticides, and others, such as parabens, is known to disrupt thyroid axis signaling, homeostasis, and function.[479-481] Evidence linking compounds such as PCBs and organochlorine pesticides to thyroid dysfunction was first observed in Great Lakes wildlife, where Herring gulls were repeatedly found with serious thyroid abnormalities and other endocrine pathologies. Since this initial observation, extensive ongoing research has been conducted that aims to link occupational and/or environmental exposures to multiple thyroid-associated diseases and pathologies.[482] Epidemiological studies support correlations of thyroid disrupting compound (TDC) exposures to adverse effects in humans and wildlife; however, direct linkages have been difficult to establish. Most epidemiological studies are supported by laboratory research, which have demonstrated multiple mechanistic targets for TDCs impacting circulating levels of thyroid hormones. As such, the most commonly used biomarker in these studies is modification of circulating serum T4 and TSH levels.[482] Thus, modifications within the HPT axis have focused on molecular/physiological events that result in altered hormone levels. However, while TSH levels are an accepted measure of hypothyroidism, a number of environmental chemicals have been demonstrated to modulate circulating thyroid hormone levels, but do not influence TSH. Additionally, it is now recognized that that several environmental chemicals interact directly as TR antagonists, which may have direct pleotropic effects.

180. The thyroid system is highly complex, and thyroid hormone homeostasis involves a complex network of homeostatic regulatory interactions.[480] TDCs have been demonstrated to target the thyroid endocrine system at multiple points within the axis. Extensive reviews have been written within the past few years, and the reader is referred to these for detailed information on mechanisms and actions of TDCs.[480; 481] Here, we provide a brief review of current targets and molecular sites of action, as these sites may be potentially useful in identifying and developing novel assays to assess and screen putative thyroid-disrupting chemicals.

181. As illustrated in **Figure 6-1**, thryroid-disrupting chemicals have been shown to target multiple sites within the HPT axis, including disruption in TRH and TSH synthesis and signaling, inhibition of iodine uptake into the thyrocyte, synthesis of T4/T3 by thyroperoxidases, modification in hormone transport-blood binding proteins, hepatic metabolism of T4/T3, disruption of deiodinase, alteration in cellular uptake/excretion of thyroid hormones, and direct interaction of compounds with the TR as direct antagonists.

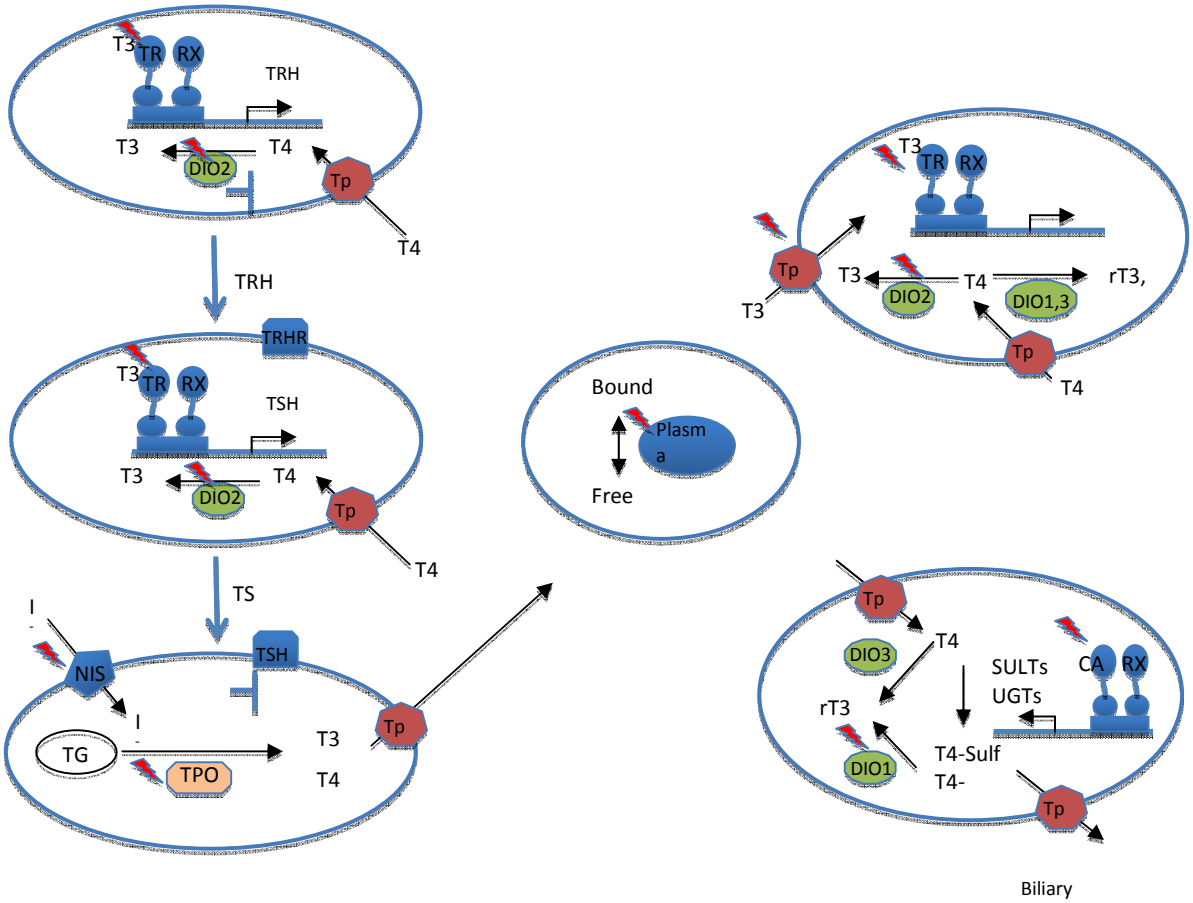

Figure 6-1. Thyroid axis and known sites of action for TDCs.

Figure modified from Jugan et al.[481] Abbreviations: TR: thyroid receptor; RXR: Retioid X Receptor; TRHR: thyrotropin releasing hormone receptor; TSHR: thyroid stimulating hormone receptor; Cytoplasmic T3BP: Cytoplasmic T3-binding protein; DIO1,2,3: deiodinases type 1, 2, 3; NIS: sodium iodide symporter; Plasma THBPs: plasma thyroid hormone-binding proteins; rT3: reverse-T3 (inactive); SULT: sulfotransferase; T4-Gluc: T4 glucuronide (inactive); T4-Sulf: T4-Sulfate (inactive); TPO: thyroperoxidase; Tpt: membrane transporter; TSH: thyrotropin; UGT, UDP, glucuronosyltransferase.

182. Thus, in addition to the previously mentioned mechanisms, there are several novel targets within this pathway that have been identified as points of action for TDCs (**Figure 6-2**). These include modification in TR expression levels; TR-RXR-TRE interaction; recruitment; binding and/or release of co-repressors; direct binding of TR agonists/antagonists to TR; binding and/or dissociation of co-activators; interference of TR-RXR heterodimerization; modification of chromatin remodeling; modifications in Pol complex recruitment; and/or polymorphic TRs affecting any of the above processes. These mechanisms have revealed that TDCs may modify transcriptional activation/repression of TR through modulation of multiple targets within the transcriptional complex and functional protein-protein or protein-DNA interactions necessary to regulate TR-mediated gene expression.

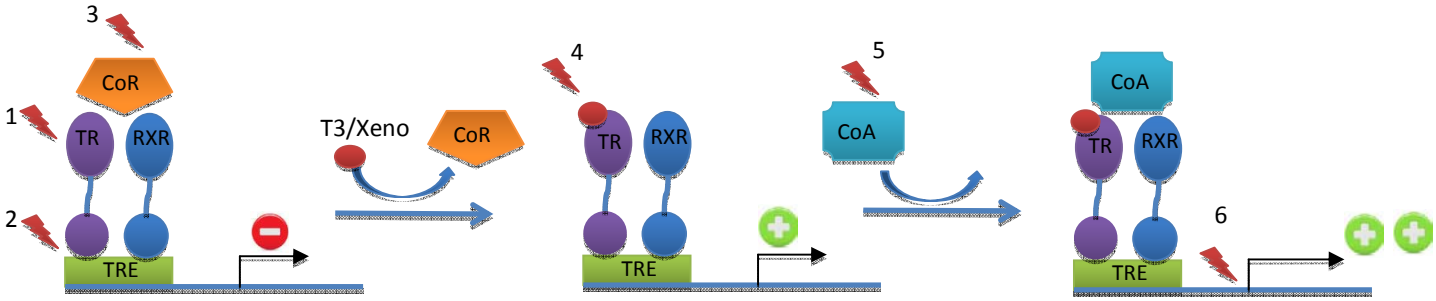

Figure 6-2. Thyroid hormone receptor transcriptional complex.

Figure modified from Jung et al.[481] 1. Expression of TR; 2. TR/RXR-TRE interactions; 3. Co-repressor recruitment, binding, dissociation; 4. TR agonist/antagonist; 5. Co-activator recruitment, binding, dissociation; 6. Chromatin acetylase/deacetylase activity, other.

183. Manipulation of thyroid hormone signaling in transgenic TR knock-out or knock-in mice has demonstrated the importance of this signaling pathway in the development of the brain,[483] bone,[484] inner ear,[485] and gastro-intestinal tract.[486] These developmental controls are elicited largely during perinatal development.[487] Thyroid hormone also functions with estradiol to regulate sexual behavior in adult female mice.[488]

184. The regulatory control of thyroid hormone on amphibian metamorphosis is well known and is the basis for the Amphibian Metamorphosis Assay (OECD TG 231). In addition, impaired thyroid hormone signaling in amphibians causes neurological defects.[489; 490] Thyroid hormone also is instrumental in development of the olfactory function in fish and amphibians and is responsible for stream recognition among salmon during smoltification.[491; 492]

6.3 Precedent Chemicals

6.3.1 AhR and CAR Agonists

185. As discussed in Section 5.3.1 (*Reductions in Retinoid Levels*), AhR ligands, such as some polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and PCBs**,** have the ability to disrupt thyroid signaling by depleting circulating thyroid hormone levels. The effect is generally considered to be due to the induction of hepatic thyroid hormone biotransformation enzymes that enhance the elimination rate of the hormone. Induction of phase one (cytochrome P450's) and /or phase two detoxification enzymes (Sult2a1 and Ugt1a1) can increase T4 clearance and lead to decreased T4 and T3 levels.[493] Comparing AhR+/- and AhR null (AhR-/-) mice, Nishimura et al.[494] demonstrated that activation of AhR by TCDD results in a marked reduction of total thyroxin and free T4 levels in mouse serum. Gene expression of *CYP1A1*, *CYP1A2,* and UDP-glucuronosyltransferase *UGT1A6* was markedly induced in the liver by TCDD and thought to be responsible, at least in part, for reduced serum thyroid hormone levels. Some PCBs are constitutive androstane receptor (CAR) activators and have been

shown, in several different studies involving various vertebrate species, to decrease T4/T3 and increase thyroid hypertrophy and TSH.[495-499] Stronger responses have been observed in females,[495] which reportedly have greater CAR levels and activity in both humans and rodents.[500-503] Interestingly, the combination of a CAR and a PPARα agonist can significantly increase thyroid hormone clearance from hepatocytes when compared to only one of the agonists.[504]

6.3.2 Deiodinase Inhibitors/Suppressors

186. In contrast, other studies suggest that CAR activation does not reduce serum T3 concentrations, but instead reduces T3 activity by inducing Dio 1, a type 1 deiodinase, which converts T4 into rT3, a much less active form of T3. Dio1 is induced by phenobarbital in a CAR-dependent manner.[505] Therefore, Dio1 induction increases rT3 (an inactive form of T3) and in turn represses T3 responsive genes such as tyrosine aminotransferase, basic transcription element binding protein, and carnitine palmitoyl transferase 1.[505] Other compounds, including FD&C red dye #3, octylmethoxycinnamate (an ultraviolet light-blocking agent used in cosmetic sunscreens), methoxychlor, and metals lead and cadmium, have also been shown to interfere with the action of the deiodinase enzymes.[482]

6.3.3 Disruptors of TSH Signaling

187. At the top of the HPT axis, TSH signaling is adversely affected by TDCs. Using Chinese hamster ovary cells (CHO) transfected with the recombinant TSH receptor, Santini et al. demonstrated that 1,1-bis-(4-chlorphenyl)-2,2,2-trichloroethane (DDT), Aroclor 1254, and lemon balm each inhibited TSH-stimulated cAMP production *in vitro.*[506] Mechanistically, lemon balm was shown to directly inhibit TSH binding, whereas the effects of DDT and Aroclor were thought to occur downstream of receptor binding. In a subsequent study, Picchietti et al. demonstrated that DDT exerts an inhibitory effect through modification of TSHr intracellular trafficking, which is necessary for TSH signal transduction.[507] Less information is available regarding the impact of TDCs on TRH signaling; however, several studies have demonstrated a significant decrease in TRH production within the hypothalamus following TDC exposure.[482] Effects on both TRH and TSH synthesis may additionally be impacted through feedback modulation of circulating T4 and T3 levels following thyroid disruption downstream of the hypothalamus and pituitary. Additionally, as described below, TDCs acting directly as TR agonists/antagonists may impact normal TRH and TSH production.

6.3.4 Disruptors of TR Signaling

188. Several TDCs directly bind to and/or suppress transcriptional activation of TH receptors (TRα and TRβ) from multiple species[480-482] *In vitro* binding assays and transactivation assays have been developed to identify thryroid-disrupting chemicals that act as either TRα/β agonists or TRα/β antagonist ligands. Chemicals with structural similarity to thyroid hormone have been the primary targets of investigation and likely candidates for TR binding.[508] Kitamura et al. investigated interaction of tetrabromobisphenol A (TBBPA), a flame retardant, and related compounds using a Chinese hamster ovary cell line (CHO-K1) transfected with human thyroid hormone receptor hTRα1 or hTRβ1.[509] In binding assays, several compounds, including TBBPA, tetrachlorobisphenol A (TCBPA), tetramethylbisphenol A (TMBPA), and 3,3'-dimethylbisphenol A (DMBPA) exhibited competitive binding with triiodothyronine. However TBBPA, TCBPA, TMBPA, and DMBPA did not transactivate the thyroid hormone-responsive reporter for either hTRα1 or hTRβ1. 2,2',4,4'-Tetrabromodiphenylether (BDE-47), also exhibits significant thyroid-disrupting activity in mammalian models, but does not exhibit hTR binding or receptor transactivation.[510] Conversely, in transient transactivation assays, TBBPA and TCBPA exhibited significant anti-thyroid hormone effects and appear to function as TR antagonists. Kojima et al. additionally screened 16 PBDEs and found only 4-OH-BDE-90 displayed antagonist activity.[511] BDE206 was also found to inhibit TR-mediated transcription.[512] Mechanistically, it is likely

that PBDEs/OH-PBDEs affect TH-regulated signal transduction pathways at multiple levels. Recently, however, Ibhazehiebo et al.[513] proposed a mechanism in which the inhibitory activity of several PDBE congeners is mediated through partial dissociation of TR from TRE *cis* elements. Some PCBs suppress thyroid hormone receptor mediated transcription.[514] A similar mechanism was proposed for where low doses of hydroxylated PCBs (OH-PCBs), including 4'-OH-PCB 106, suppressed thyroid hormone-mediated transcription through partial dissociation of TR from TRE.[515] This dissociation was observed on both artificial TH-response elements, such as direct repeat (DR)-4, and native TRE-containing promoters, such as malic enzyme (ME)-TRE.[516] It thus appears that both PBDEs and OH-PCBs may modulate receptor transactivation in a similar fashion.

189. Recent *in vitro* studies have also demonstrated that dibutyl phthalate (DBP), monobutyl phthalate (MBP), and di-2-ethylhexyl phthalate exhibit potent TR antagonist activity.[517] Both DBP and MBP enhanced protein-protein interactions between TR and the nuclear receptor co-repressor SMRT (silencing mediator of retinoid and thyroid hormone receptors in a mammalian two-hybrid assay.[518] The functional significance of this interaction is to be determined, but in some instances, nuclear receptor interaction with transcriptional co-repressors may lead to enhanced TR- regulated gene transcription.[519] Other studies have identified additional sites of action in which TR transactivation may be disrupted by TDCs. These mechanisms are detailed in Jugan et al.[481] and briefly reviewed here (see Figure 6-2). Regulation of TR mediated transcription involves a progression where, in the absence of T3, the TR and RXR heterodimerize and bind to a thyroid response element (TRE) on DNA. Recruitment of nuclear receptor transcriptional co-repressors, such as SMRT or NCOR, represses basal transcription through chromatin deacetylase activity. T3 binding to TR causes the release of the co-repressor and restores basal activity. Subsequent recruitment of nuclear receptor co-activators (SRC-1, SRC-2 and others) destabilizes chromatin and enhances transcriptional activity through histone acetylation and contacts with the basal transcriptional machinery.[520; 521]

6.3.5 Disruptors of Iodine Uptake and Thyroid Hormone Synthesis

190. The effect of TDCs on the NIS receptor protein has been illustrated with several environmental chemicals, including, perchlorate, thiocyanate, bromate, and nitrate.[522] Each of these compounds compete with iodine for binding to the NIS transport protein inhibiting the uptake of iodine into the follicular thyroid cell.[523] PCBs, on the other hand, down regulate expression of NIS.[524] The putative effect of this inhibition/down regulation is a decreased synthesis of T4 and T3. Also, within the follicular thyroid cell, certain TDCs, including mancozeb (fungicide), amitrole (herbicide), ethylenethiourea (a fungicide metabolite of bisdithiocarbamates), soy isoflavones, and benzophenone 2, inhibit formation of thyroid hormones and/or activity of TPO. Inhibition of TPO impedes the ability of the follicular cell to synthesize T4 and T3.[480]

6.3.6 Disruptors of Plasma and Cross-Membrane Transport Proteins

191. TDCs may also impact circulating levels of free and bound thyroid hormones through their ability to bind with thyroid hormone transport proteins. Some PCBs, flame retardants, phthalates, and penta-chlorophenol each bind to TTR. In their bound form, these chemicals compete with thryroid hormones modifying ratios of free to bound hormone. Additionally, chemicals bound to TTR and TBG may be transported to normally inaccessible sites of action, including fetal compartment and fetal brain, with a resultant decrease in fetal brain T4 levels.[525] Some PCBs, flame retardants, dioxins, and bisphenol A modulate active transport and cellular uptake of thyroid hormones through disruption of hormone cross-membrane transport proteins, including monocarboxylate transporter 8 (MCT8) and organic anion transport protein (OATP).[480; 482] Richardson et al., found that PBDEs directly modify mRNA expression of (MCT8).[499] These and other studies suggest that exposure to TDCs may alter mechanisms associated with hormone uptake and biliary excretion.

6.4 In Vitro Assays

192. Zoeller and Tan[526] reviewed existing guidelines and strategies for thyroid screening and testing and provided an assessment of those assays that could adequately capture the range of targets within the thyroid endocrine system that may be disrupted by these toxicants across vertebrate taxa.[526] While some of these assays have been developed and validated for use by OECD, others were not further assessed for inclusion as validated screening assays.

193. The goal of this section is to provide a current update to the state of recent additions and modifications of novel assays to assess and screen thyroid-disrupting compounds. Assays included here represent either development of novel mechanisms to assess HPT disruption, or modifications of previously described assays for higher throughput assessments. Information included in this update represents all current assays and methods currently listed in the published literature between 2008 and 2011.

6.4.1 Transactivation Reporter Assays with TRα and TRβ

194. Numerous studies have employed transient transfection assays to screen compounds for TR agonist and/or antagonist activity. The basis of this assay consists of transient expression of TRα or TRβ cloned into a mammalian expression vector (pCDNA, pSG5, or other) containing a strong constitutive promoter such as CMV or SV40. Receptor constructs are transfected into a mammalian cell lines (monkey fibroblast-derived CV-1 or human medulloblastoma-derived TE671) with low endogenous expression of either TR receptor form. A reporter gene, usually luciferase, under genetic regulation of a native or synthetic TR responsive promoter containing one or more TREs, is co-transfected and used for quantitative assessment of transactivation activity. Plasmids containing either *Reniella luciferase* or β-galactosidase are additionally co-transfected for normalization between replicate wells and between assays. Assays are conducted in 24, 48, or 96 well plates, and scale-up for high-throughput assessment can easily be obtained. Some assays additionally titrate RXR, the TR receptor heterodimerization partner, and /or nuclear receptor co-regulators, such as SRC-1, or PGC1α to enhance transactivational activity of the assay.

195. Transfections using either empty vector or an absence of ligand may serve as a control for basal activity of the reporter gene. T3 is used as a positive control for the assay and induces luciferase activity as a concentration-dependent factor between 10^{-10} to 10^{-6} M. Dose–response analysis at these concentrations suggests that the assay is highly sensitive, with an approximate T3-EC_{50} of 1.50×10^{-8} M, and maximal induction of 346-fold can be achieved at concentration of 10^{-6} M T3.[517] Shen et al. demonstrated that, at 10^{-6} M T_3, induced luciferase activity with an intra-assay within coefficient of variation (CV) of 7.4% and the inter-assay CV of 18.5%.[517] Compounds can be tested for either agonist and/or antagonist activity. Antagonist activity is assessed through competitive inhibition of transcriptional activity in the presence of T3. In general, use of this assay with both native and synthetic TRE-containing promoters has demonstrated that most compounds tested do not function as TR agonists. Early use of this assay demonstrated that several PCB congeners, including OH metabolites such as 4-OH-PCB-106 and a PCB mixture (Aroclor 1254), suppress TR-mediated transcription.[515] Antagonistic activity has also been observed with multiple compounds, including OH PCBs, dioxins, and phthalates.[510; 527] For example, DBP, MBP, and DEHP possessed antagonist activity with IC_{50} of 1.31×10^{-5}, 2.77×10^{-6} M and exceeding 1.0×10^{-4} M, respectively.[518]

196. It should be noted, however, that *in vitro* studies with either transient or stable expression of TH receptors in cell systems are predominantly limited to assessment of parent compound examined. Receptor transactivation only reflects primary ligand binding unless cells are "metabolically" active. Thus, care should be taken when interpreting results as metabolic activation or inactivation may not be

accounted for. As an example, when phthalates are ingested (the most common route of human contact), they are converted to mono-esters and usually absorbed in that form; significant systemic levels of parent (di-ester) forms of these molecules occur rarely and only under some restricted circumstances. Accordingly, results of *in vitro* screening tests of parent (di-ester) phthalates can be very misleading[246] and irrelevant to the *in vivo* situation.

197. Particular care should also be taken in order to standardize controls and cell growth in transient transfection experiments. Using empty vectors as a control for reporter genes can be problematic as copy numbers can differ compared to inserts containing vectors. Reasons for this are the metabolic load of the insert, as well as the higher replication efficiency of smaller plasmids. Further, copy numbers may vary during cell growth in batch systems.

6.4.2 Two-hybrid Assays

198. The yeast two-hybrid assay has also been employed to assess for TR ligands. This assay system is based on the ligand-dependent interaction between nuclear hormone receptor and nuclear hormone receptor co-activators. Nuclear receptor-based yeast two-hybrid assays for TR ligand interactions were initially reported by Hawkins and Thomas.[151; 528] The fundamentals of the assay include development of two fusion proteins, including the yeast GAL4 DBD, with the nuclear receptor LBD, GAL4(DBD)-NR(LBD) and a second fusion protein consisting of the GAL4 DBD with the nuclear receptor co-activator LXXLL motif-interaction domain GAL4(DBD)CR(AD). Both fusion proteins are expressed in a yeast strain, which harbors a GAL4 DNA binding site upstream of a *lacZ* reporter gene. In the presence of ligand, the GAL4DBD-nuclear receptor fusion protein binds to the GAL4 response element within the promoter region of the *lacZ* gene. Once bound, the GAL4DBD-nuclear receptor interacts with GAL4AD-co-activator, which recruits the basal transcriptional machinery to the promoter region of *lacZ* gene, resulting in production of β-galactosidase. The β-galactosidase activity level corresponds to the strength of both the TR-ligand interaction and the interaction between TR and the coactivator. Using a yeast two-hybrid system containing human TRα and the coactivator, transcriptional intermediary factor (TIF2), Kitagawa et al. found a lower limit of T3-TR binding activity in this assay to be 3.0 X 10^{-8} M and a calculated EC10 of 1.0 X 10^{-6} M.[529] Comparatively, assessments of relative binding efficiencies for several TDCs suggested that phenolic hydroxyl groups and *ortho*-substituents may be important structural features for TR interaction. Numerous improvements have been incorporated into the yeast two-hybrid system, including addition of a rat liver S9 metabolic component and enhanced detection sensitivity by adapting β-galactosidase detection to chemiluminescence.[530; 531] In an assessment of the thyroid hormone activity of a series of monohydroxy PCBs, Shiraishi et al.[530] incorporated rat liver S9 fraction in the yeast two-hybrid assay to determine necessity of metabolic activation prior to TR binding. Chemicals were first incubated with rat liver S9 fraction, followed by addition of yeast to the assay system. Similarly, Li et al.[400] developed a yeast two-hybrid assay using the human TRβ/GRIP coactivator system. TDC antagonist activity was assessed in the presence of 5.0 X 10^{-6} M T3, which induced maximal β-galactosidase activity. Results of this study identified two partial TRβ agonists, including 2-*t*-butylphenol and 2-isopropylphenol. The remainder of compounds screened exhibited partial antagonist activity, with 20% relative inhibitory concentration greater than 10^{-7} M; however, PHAHs exhibited RIC20 values less than 5 x 10^{-7} M following incubation with rat liver S9 fraction. More recently, Terasaki et al.[531] demonstrated that halogenated derivatives of BPA, 3,3',5,5'-tetrabromobisphenol A (TBBPA), 3,30,5,50-tetrachlorobisphenol A (TCBPA), and 3,30,5-trichlorobisphenol A (3,3',5-triClBPA) exhibited partial TRα agonist activity prior to metabolic activation. Subsequent to incubation with rat liver S9 fraction, the activities of TBBPA and TCBPA increased markedly (7.6-fold and 3.1-fold, respectively) whereas other halogenated BPA derivatives inhibited the binding of triiodothyronine (T3) to TRα at 2 X10^{-5} M without rat liver S9 treatment and at 4 x 10^{-6} M with rat liver S9 treatment, demonstrating their T3 antagonist activity.

199. Mammalian two-hybrid systems have been useful to screen nuclear receptor-nuclear receptor co-regulator interactions. These assays are conducted as a transient expression assay, where mammalian cells such as green monkey kidney fibroblast (CV-1) are transfected with expression plasmids containing a fusion protein consisting of the yeast GAL4 DNA binding domain and the interaction domain (LXXLL) of a nuclear receptor co-regulator (coactivator or co-repressor), a VP16-hTR fusion protein, and a GAL4 responsive luciferase reporter such as pUAS-tk-luc. Following transfection, cells are treated with compounds of interest and examined for ligand-dependent recruitment of nuclear receptor and co-regulator interactions. The relative transcriptional activity is converted to fold induction above the corresponding vehicle control value.

200. Using data from transient transactivation assays, several studies have demonstrated that TDCs can suppress transcriptional activation of TR-mediated gene expression. To investigate the mechanisms of this suppression, multiple investigations have turned to mammalian two-hybrid assays to assess if TDCs can either facilitate or modulate coregulator (coactivator and/or corepressor) interaction with TR. Investigations into the mechanisms of DBP and MBP, Shen et al. demonstrated that DBP and MBP enhanced the interactions between co-repressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) and TR in a dose-dependent manner.[517] Conversely, Ibhazehiebo et al. found that polybrominated biphenyl mixture BP-6 did not alter recruitment of corepressors to TR or inhibit coactivator binding to TR in the presence of ligand.[532] Similarly, PBDEs did not alter ligand-dependent cofactor (SRC-1) recruitment to TRβ1.[533]

6.4.3 DNA Binding Assays

201. Traditionally, assessment of nuclear receptor DNA interactions is conducted using an *electrophoretic mobility shift assay* (EMSA). Recently, however, a novel liquid chemiluminescent DNA pull-down assay has been developed to rapidly assess TR-DNA (TH response element [TRE]) binding.[142] This assay measures nuclear receptor-DNA binding in solution and shows great promise for high-throughput assessment of this mechanism of TDCs disruption. Briefly, a glutathione S-transferase (GST)-fused TR protein is bound to glutathione-sepharose beads and incubated with a digoxigenin-labeled double-stranded DNA fragment containing a TRE. After repeated washing, protein–DNA binding on sepharose beads is detected using anti-digoxigenin antibody conjugated to alkaline phosphatase, which is then measured by a chemiluminescent reaction using a luminometer. Using this approach, Ibhazehiebo et al.[533] discovered that repression in transactivation of TR following exposure to polybrominated biphenyls and PBDEs is due to partial dissociation of TR from TRE.

6.4.4 Dendritic Arborization

202. TRs are ubiquitously expressed in most cerebellar cells, including Purkinje cells, during development, and previous studies have demonstrated that TH induces Purkinje cell dendrite development in rodents via TR gene transactivation.[534; 535] Several studies have thus investigated the impact of TDC exposure on TH-dependent dendrite arborization of cerebellar Purkinje cells. This assay requires isolation of primary rat or mouse Purkinje cells, as described by Kimura-Kuroda et al.[537], and subsequent exposure to test compounds of interest in the culture media for 17 days. Dendrite arborization is assessed through immunocytochemical staining for calbindin using mouse-monoclonal anti-calbindin-28 K primary antibody and a fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse IgG secondary antibody. Immunolabeling is observed under a laser confocal scanning microscope, and the extent of arborization is quantified by tracing the outline of the cell and dendritic branches of randomly selected Purkinje cells and computing the area using imaging software (NIH). Numerous studies have used this assay to test the ability of TDCs to disrupt dendrite arborization following TDC exposure. Kimura-Koroda et al.[536] first demonstrated that 4-OH-2',3,3',4',5'-pentachlorobiphenyl and 4-OH-2',3,3',4',5,5'-hexachlorobiphenyl significantly inhibited the TH-dependent extension of Purkinje cell dendrites, even at extremely low

concentrations. Subsequently, the same group demonstrated that additional OH-PCB's and BPA significantly inhibited the TH-dependent dendritic development of Purkinje cells, whereas other PCB metabolites progesterone and nonylphenol significantly promoted the dendritic extension of Purkinje cells in the absence of THs.[537; 538] More recently, Ibhazehiebo et al.[539] demonstrated that PBDE, 1,2,5,6,9,10-αHexabromocyclododecane (HBCD) and a polybrominated biphenyls (PBBs) mixture PB-6 significantly suppressed TH-induced Purkinje cell dendrite arborization.[532; 533]

6.4.5 Neurite Extension

203. As with Purkinje cells, TRs are ubiquitously expressed in most cerebellar neuronal cells, including granule cells during development.[534] Mouse cerebellar granule cell have been used extensively as a model system for studies on mammalian central nervous system neurogenesis.[540] Their characteristic morphology, cell size, and large numbers during early postnatal development in rodents allow for their purification for *in vitro* analysis and, thus, neurite extension is becoming an additional cell-based assay to investigate the impact of environmental chemicals on thyroid-mediated neuronal development. The assay is dependent upon the isolation and purification of rat cerebellar granule cells according to the methods of Okano-Uchida et al. and analysis of granule cell neurite extension is conducted in real time using a light microscope with a charged couple device (CCD) video camera and cell imaging software.[532; 541] The impact of TDC exposure is assessed through measuring TH-mediated granule cell neurite extension and elongation. In the presence of T3, granule cell aggregates form elaborate tree-like neurites with several secondary shafts and bifurcating branches, while those without T3 exhibit limited neurite extension and have fewer bifurcating branches.[513] With addition of TDCs, including low doses (10^{-10} M) of HBCD, TH-induced neurite growth and extension of the granule cell aggregate is significantly suppressed, with markedly reduced length and secondary branches and bifurcations poorly developed resulting in reduction of total neurite granule cell area. In a similar study, Ibhazehiebo et al. demonstrated that addition of a PCB mixture PB-6, greatly impaired neurite growth and extension, including size, number, length, and area of neurites of the granule cell aggregate.[532]

6.4.6 Cell Proliferation Assay

204. The "T-screen" is a cell proliferation–based assay used for the *in vitro* detection of TR agonists and antagonists.[542] GH3 cells used in the T-screen assay are derived from a rat pituitary tumor cell line. GH3 cell growth is dependent on the thyroid hormone T3 and mediated by high levels of expression of TR in the cell. The assay specifically measures cell proliferation following exposure to T3. Interaction of xenobiotics with the TRs and/or the TR transcriptional complex may result in agonistic effects on cell growth, whereas interactions of antagonists result in inhibition of T3-induced cell growth. Cell proliferation is determined by measuring the total metabolic activity of GH3 cells using the dye resazurine.[543] Enzymes in the mitochondria of GH3 cells reduce oxidized blue resazurine to the highly fluorescent complex resorufin. Fluorescence intensity is a measure of the quantity of viable cells present. Cell proliferation is expressed as a mean percentage of the maximum T3-induced effect (set at 100%).

205. Initial studies utilizing the T-screen assay investigated a series of specific TR agonists and antagonists and made significant modifications to the assay to optimize it for fast and inexpensive screening of T3-like activity. These optimizations include the replacement of alamarBlue® used in the previous study by the much cheaper resazurine, and the use of fetuin as an additional growth factor to the medium to stimulate attachment, spreading and growth of the GH3 rat pituitary cells when cultured in serum-free medium. Subsequently, the T-screen has been used to assess TDC activity of many compounds, including PCBs,[544] PAHs,[512] nitrates,[545] and others. Many *in vitro* systems do not reflect metabolic conversion of parent compound to putative TDCs. To address this issue, modifications can be made to the T-screen that include incorporation of a metabolic system to the assay. Taxvig et al. tested both the human liver S9 mix and the PCB-induced rat liver microsomes to determine possible changes in

the ability of the TDCs to bind and activate the thyroid receptor in the T-screen assay after biotransformation.[546] Using parabens and phthalates as target compounds, the authors found no marked difference in cell proliferation between the parent compounds and the effects of the tested metabolic extracts. Assessment of GH3 cells alone suggests that these cells have some metabolic capabilities. Results from this study suggest that an *in vitro* metabolizing system using liver S9 or microsomes could be a convenient method for the incorporation of metabolic and toxicokinetic aspects into *in vitro* testing for endocrine-disrupting effects in this system.

206. Schreiber et al.[547] also employed primary fetal human neural progenitor cells (hNPCs), which are cultured as neurospheres to mimic basic processes of brain development *in vitro*. This assay examines proliferation, migration, and differentiation of hNPCs following treatment to desired TDCs. The assay encompasses growth of normal human neural progenitor cells cultured as free-floating neurospheres in proliferation medium and plating onto a poly-D-lysine/laminin matrix. Assessment of cell viability, migration, and differentiation of neurospheres is conducted following a 1- to 2-week preincubation period with test compounds. Cell proliferation is determined by measuring sphere size. Migration is measured by determining the distance from the edge of the sphere to the furthest migrated cells 48 hours after initiation of differentiation at four defined positions per sphere. Cell proliferation is determined through changes in cell number by measuring sphere diameter in contrast to the negative control without mitogens. Cell viability is measured using the alamarBlue assay (which measures mitochondrial reductase activity). Assessment of PBDEs in this assay revealed that these compounds do not disturb hNPC proliferation, but rather decrease migration distance of hNPCs. Moreover, PBDEs result in a marked reduction of differentiation into neurons and oligodendrocytes.

6.4.7 Thyroid Peroxidase (TPO) Inhibition Assay

207. TPO is a heme protein localized in the apical cytoplasmic membrane of thyroid epithelial cells and plays an important role in thyroid hormone biosynthesis.[440] Specifically TPO facilitates the organification of iodide and the iodination of tyrosyl residues of thyroglobulin (Tg). TPO inhibition is a target for propylthiouracil (PTU) and methimazole (MMI); currently, the only antithyroid drugs with known therapeutic relevance for the treatment of hyperthyroidism.[548] Schmutzler et al. developed a novel *in vitro* assay based on human recombinant TPO (hrTPO) stably transfected into the human follicular thyroid carcinoma cell line FTC-238.[549] The FTC-238/TPO cells are used as a source of hTPO. Functional hrTPO is prepared by digitonin extraction of the cell membranes from FTC-238/TPO cell and assessed *in vitro* for peroxidase activity using the guaiacol oxidation assay, as previously described.[550] TPO activities are calculated as micromole H_2O_2 reduced per minute and per milligram protein. TPO inactivation assay are conducted by preincubating protein extracts with selected compounds followed by assessment of peroxidase (guaiacol assay) activity.

208. In this system, several suspected TDCs from plant sources inhibited TPO activity, including genistein, resveratrol, silymarin, and the synthetic flavonoid F21388. Screening of industrial chemicals revealed that 4-nonylphenol and BPA also inhibited TPO, with IC50 values ranging from 0.83 to 174 µmol/L, whereas compounds including 4-MBC, procymidon, linuron, BP3, 4-nonylphenol, and estradiol, had no effect on TPO activity.[548]

209. Partially purified hog TPO has also been used as an abundant source of enzyme in TPO inhibition assays and there is a need for a critical evaluation of the various TPO substrates from multiple species. For ethylenethiourea and N,N,N',N'-tetramethylthiourea (TMTU), millimolar concentrations are necessary in order to achieve some inhibition, if guaiacol is used as a substrate, whereas with iodide as the substrate low, micromolar concentrations of ethylenethiourea and TMTU are sufficient to temporarily suppress iodination or the formation of the iodide trianion (I^{3-}).[551; 552] The presence or absence of iodide is

also linked to a shift in the mode of action for compounds like PTU or MMI (i.e., irreversible inhibition of TPO in the absence of iodide, temporary suppression of iodination in the presence of iodide).[553]

6.4.8 Iodide Uptake Assay

210. Iodide accumulation in the epithelial cells of the thyroid gland is the first step in thyroid hormone biosynthesis. This process is catalyzed by the sodium-iodide symporter (NIS), a member of the SGLT-1 sodium glucose cotransporter type 1 family of sodium-dependent transporters. NIS–iodide uptake activity has previously been determined using nontransformed rat thyroid cell line FRTL-5.[548] This assay incorporates growth of the FRTL-5 cells to confluence and assessment of iodide uptake in the presence of selected test compounds to detect direct interference with NIS function. NIS activity is measured by incubating cells in HBSS and and media containing ^{125}I and determining cellular uptake of radioactive iodide. Results are calculated as the amount of iodide accumulated per microgram of protein. Results from this assay demonstrate that several compounds inhibit NIS activity, including the soy isoflavone genistein, UV filters, 4-MBC, 4-NP, and perchlorate.[548] One compound tested, xanthohumol, exhibited stimulation of iodide uptake by NIS at nanomolar concentrations.

6.4.9 Thyroid Hormone Binding Protein Assays

211. Several studies have shown that *in vivo* exposure of experimental animals to TDCs results in reduction of the T4 level in serum due to TDC binding with thyroid hormone transport proteins and displacement of T4.[554] To investigate the binding interactions of TDCs with hormone-binding proteins, Cao et al. utilized a novel fluorescence displacement method.[555] The assay incorporates a protein-binding fluorescence probe that is not fluorescent when free in solution, but becomes highly fluorescent after its binding to a protein. If an analyte binds to the protein at the same site as the probe, it will displace the probe from the protein and reduce the fluorescence intensity. From an analyte titration curve, an IC_{50} value can be obtained, and the binding constant of the analyte with the protein can be calculated. In this assay, 8-anilina-1-napthalenesulfonic acid (ANSA) is used as the fluorescence probe due to its known iteraction with TTR and TBG. Assessment of 14 OH-PBDEs with this assay demonstrated that these compounds competitively bind to both TTR and TBG, in the range of $1.4 \times 10^7\ M^{-1}$ and $6.9 \times 10^8\ M^{-1}$ for TTR and between $6.5 \times 10^6\ M^{-1}$ and $2.2 \times 10^8\ M^{-1}$ for TBG.

6.5 In Vivo Assays

6.5.1 Modification of Long Term In Vivo Assays

212. Multiple long term *in vivo* bioassay methods that include thyroid-related endpoints have been developed in a variety of species, including rat (e.g., OECD TGs 407, 416) fish (Fish Two Generation), and amphibians (Amphibian Metamorphosis Assay [21 day]); for a complete list, see those previously described by Zoeller et al.[440] The relatively conservative nature of many components of the HPT axis among vertebrates suggests that extrapolation of chemical effects among different species may be feasible.[526] *In vivo* approaches, however, are inherently lengthy and often costly; thus, recent efforts have focused on enhancing these model systems for TDC screening purposes, including development of shorter bioassays with more diagnostic endpoints. To achieve this goal, early temporal responses, including gene expression and histological changes, are being incorporated into these and additional assays and compared to results obtained in long-term studies.

213. Tietge et al. reported using a short term *Xenopus laevis* assay examining thyroid gland histology and cell numbers, circulating TH concentrations, and thyroidal TH and associated iodo-compounds throughout an 8-day exposure to three TH synthesis inhibitors: methimazole (100 mg/L), 6-propylthiouracil (20 mg/L), and perchlorate (4 mg/L).[556] Results from this assay were observed within

2–6 days of exposure and indicative of inhibitory effects of the chemicals on TH synthesis. Similarly, perchlorate and ethylenethiourea exposed *X. laevis* larvae were assessed for selected transcriptional responses within 3–5 days of exposure. These results support the concept that shorter-term *in vivo* assays are feasible and can recapitulate some of the more long-term endpoints of the amphibian metamorphosis assay.[557]

214. Numerous studies are now incorporating transcriptional responses as short-term measures of *in vivo* HPT axis disruption. These include assessment of tissue-specific responses in thyroid, brain, liver, and other TR peripheral tissues. TR gene targets are selected a priori based upon known mechanisms of thyroid hormone function. For example, Wang et al. recently examined gene expression differences for target genes, including BTEB, TRβ, BDNF, GAP-43, and NCAM1 in rat brain following gestational exposure to perfluorooctane sulfonate (PFOS) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) on post-natal days 1, 7, and 14.[558]

215. Gene expression changes have also been assessed in non-mammalian models, including *Xenopus* and zebrafish. For example, Shen et al. reported gene targets, including TRβ, RXRγ, and TSHα and TSHβ, were each modified following exposure to DBP and MBP in *Xenopus*. Similarly, the chemical-induced effects impacting cross-talk between the HPG, HPA, and HPT axes of prochloraz or propylthiouracil (PTU) exposed adult zebrafish were examined using a 20 gene qPCR array.[559]

216. Multiple studies have additionally applied a microarray and other transcriptomic approaches to assess global gene expression changes following TDC exposures *in vivo*. Heimeier and Shi[560] used a microarray approach to anchor BPA-induced gene expression changes with intestinal remodeling in premetamorphic *Xenopus* tadpoles.[534] Importantly, microarray analysis revealed that BPA antagonized the regulation of most T3-response genes, thereby explaining the inhibitory effect of BPA on metamorphosis. Similarly, Ishihara et al. used gene expression profiling to examine the thyroid hormone-disrupting activity of hydroxylated PCBs in metamorphosing amphibian tadpole.[561] They concluded that genome-wide gene expression analysis in *Xenopus* brain following short-term exposure could be coupled with bioinformatics to provide an overview of the molecular mechanisms underlying thyroid-disrupting activities *in vivo*.

6.5.2 Organ Culture

217. Several groups have proposed *ex vivo* thyroid explant assays as a means to assess TCDs directly on thyroid physiology and gene expression. Hornung et al. recently developed assays to directly investigate chemicals for thyroid hormone disruption using thyroid gland explant cultures from *X. laevis* tadpoles.[562] These assays are similar to high-throughput, cell-culture-based assays in that they are conducted in 96-well plates. This assay functions similar to *in vivo* assays in that the cultured thyroid gland retains the functional integrity and natural response to TSH necessary for thyroid hormone synthesis and secretion. In brief, thyroid gland explant cultures from prometamorphic *X. laevis* tadpoles are isolated and assessed for chemical induced thyroid hormone synthesis disruption. Thyroid glands are continuously exposed for 12 days to each compound, and T4 hormone synthesis is measured daily. The potency of compounds to inhibit T4 release is determined using glands co-treated with a single maximally effective bTSH concentration and graded concentrations of chemical.

218. A similar approach was used by Schriks et al. where an *X. laevis* tadpole tail tip regression assay was used as a bioassay to detect thyroid hormone disruption.[563] The basis of this assay stems from the observation that thyroid hormone regulates amphibian metamorphosis, including regression of the tail. In this assay, tail tip regression is shown to be highly responsive to T3. The ability of TDCs to antagonize and/or potentiate this response can be tested by treating tail tips *in vitro* to selected chemical agents in the presence or absence of T3. In this study, tail tips were exposed to two brominated flame retardants

(BFRs). T3-induced tail tip regression was antagonized by 2,2',3,3',4,4',5,5',6-nona brominated diphenyl ether (BDE206) and potentiated by hexabromocyclododecane (HBCD) in a concentration-dependent manner, which was consistent with results obtained with T-screen assay. The bioassay proved to be suitable not only for detecting T3-agonists, but also for antagonists and potentiation. A similar tail regression assay was used to assess the impact of arsenic on thyroid hormone-mediated amphibian tail metamorphosis.[564]

6.5.3 Additional In Vivo Models

219. Modifications to existing assays and development of novel *in vivo* assays have enhanced TDC screening *in vivo*. Particular advancement has been made in non-mammalian models, including zebrafish and *Xenopus*. In 2009, Raldua and Babin reported the development of a simple, rapid zebrafish larva bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function.[565] This assay was designed to incorporate European and United States policies for the development of simple methodologies for screening endocrine-disrupting chemicals. In this assay, zebrafish are used as a model organism to detect the potential effects of TDCs on thyroid function. This method uses a T4 immunofluorescence quantitative disruption test (TIQDT) to measure thyroid function. The basis of the assay examines the impact of TDC to abolished T4 immunoreactivity in thyroid follicles of zebrafish larvae.

220. Transgenic reporter animals additionally have the potential to be incorporated into *in vivo* TDC screening protocols. Terrien et al. [566] recently studied the effects of such TDCs *in vivo* using transient transgenic zebrafish (*Danio rerio*), expressing Green Fluorescent Protein (GFP) under the control of the *Xenopus* TH/bZIP promoter. Exposure of this line to T3, a T3 signaling agonist (TRIAC), a TR antagonists (NH(3) or NaClO(4)), or to the endocrine disruptor BPA modified GFP fluorescence in both F0 embryos and larvae. The zebrafish transgenic line was established based upon previous studies conducted in *Xenopus*, with the aim of developing a physiological system compatible with high-throughput analysis. In 2007, Fini et al. reported development of a high-throughput method to assess potential effects of EDCs in *Xenopus in vivo.*[567] The aim of this approach was to identify an assay that would provide the full spectrum of physiological impacts exerted by a given chemical. The authors developed fluorescent transgenic *X. laevis* embryos bearing a TH/bZIP-eGFP construct that could be conducted in 96-well plates. The system incorporates NF-45 embryos and allows rapid detection of chemical interference with both peripheral TR signaling and production of endogenous TH and has a low assay variability.

6.5 Strengths, Challenges and Limitations

221. A clear precedent has been set for the ability of TDCs to disrupt multiple targets within the HPT axis. Mechanistic studies have established defined sites of action for TDCs, which have subsequently been exploited for development of defined assay systems, including direct interaction of xenobiotics with thyroid hormone receptors, TPO enzyme activity, NIS activity, and others. Continued identification of novel TDC targets is likely to advance the ability to develop screening assays and further our understanding of the biological actions of TDCs. As an example, the recent discovery that PCB and PBDEs cause TR-TRE dissociation resulted in development of a chemiluminescent DNA pull-down assay to rapidly assess TR-DNA /TRE response element binding-interactions. This *in vitro* assay, as well as others discussed, has the potential to be adapted to TDC screening assays with the potential for large-scale screening for this mechanism. Thus, several newly developed assays show promise as valuable tools for identification and quantification of compounds active in disturbing thyroid hormone homeostasis. Modifications to *in vivo* assays will additionally play a significant role in assessment of TDC activity. As multiple long-term assays have been developed and undergone OECD validation, incorporation of short-term components such as gene expression and histological changes to these assays will prove beneficial.

Key to these developments, however, will be the necessity to demonstrate that short-term end points are predictive of apical, long-term consequences of TDC exposures and that correct estimates for target organ doses and the testing of metabolites for potential endocrine activities can be determined. Additionally, use of non-mammalian vertebrate *in vivo* models, including zebrafish (or other fish species) and *Xenopus*, will significantly aid to the battery of screening options. Specifically, the use of reporter species that can be modified to fit 96- or 384-well assays will prove to be highly advantageous for *in vivo* assessment of TDCs. This is particularly important when designing a screening system that demands both rapid throughput and an intact physiological system.

Table 6-1. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on the thyroid hormone signaling pathway*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: TR activation/inhibition; AhR activation, CAR activation T4, iodine displacement TR-DNA binding interference	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	TR transactivation reporter assay; AhR, CAR reporter assays; Thyroid peroxidase assay, Iodine uptake assay, T4 binding protein displacement assay; EMSA, DNA pull-down assay
Tissue-level responses Neuronal cell development and proliferation Pituitary cell proliferation Thyroid gland function Tissue responsiveness to TH	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (Relevant cell-based assays were included in this level)	Dendritic arborization assay, Neurite extension assay, Neural progenitor cell proliferation assay; T-screen assay; TH production in thyroid gland explants; Tadpole tail explant resorption assay
Organ-level responses Thyroid gland histology	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	Endpoints present in male and female pubertal rat assays
Whole organism responses Frog metamorphosis Thyroid development Thyroid hormone levels	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Existing level 5 assays are adequate to assess whole organism apical effects relevant to thyroid hormone signaling disruption

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

222. It is likely that any screening process for thyroid hormone disruption will incorporate a battery of both *in vitro* and *in vivo* assays. The above descriptions, in conjunction with use of an AOP approach

(**Table 6-1**), should provide a foundation upon which the best approach for developing an appropriate complement of TDC screening assays can be determined. The AOP will assist in determining key events in the HPT pathway and facilitate identification of an appropriate complement of assays to query disruption. Large screening protocols for multiple compounds will likely initially utilize a complement of *in vitro* assays to identify putative TDCs. These initial screens will likely be followed by subsequent short term *in vivo* assessments that incorporate ADME considerations and are amenable to scale-up. Validation of HPT disruption is likely to be conducted using longer-term *in vivo* assays.

THE VITAMIN D SIGNALING PATHWAY

7.1 Overview

223. Vitamin D is a steroid hormone. Like other members of this family, the biological effects of vitamin D are mediated through the binding of 1α, 25-dihydroxyvitamin D_3 to its hormone receptor, vitamin D receptor (VDR). VDR is a member of the nuclear receptor superfamily, which makes up a large group of ligand-activated transcription factors. The mechanism of VDR-mediated gene transcription closely resembles that of other steroid hormones. 1α, 25-dihydroxyvitamin D_3 binds to the ligand-binding pocket of VDR with high affinity ($K_D = 10^{-10}$ to 10^{-11} M).[568] The binding of VDR to its ligand causes a conformational change in the receptor to its active form. VDR heterodimerizes with RXR, and the heterodimer binds to target genes containing a canonical vitamin D response element (VDRE) within the promoter region.[569] Co-regulatory proteins are recruited, followed by the recruitment of RNA polymerase II and the initiation of gene transcription.

224. Vitamin D is an ancient molecule that is found in animals, plants, and zooplankton. While vitamin D is ubiquitous among organisms, VDR is only found in vertebrates.[570] VDR and vitamin D signaling likely originated with stem vertebrates, as a functional VDR has been identified in the sea lamprey (*Petromyzon marinus*), a basal vertebrate lacking a calcified skeleton.[571; 572] Comparisons of vertebrate VDR protein sequences demonstrate a high degree of conservation across species, suggesting that the vitamin D endocrine axis may be highly conserved throughout vertebrate evolution.[573] In humans and rodents, 36 tissues express VDR, including tissues that are not associated with the classic vitamin D effects of calcium mobilization and ion homeostasis.[569] In fact, recent investigation of VDR function suggests that VDR signaling has additional non-calcemic roles, including roles in immune system function, cell proliferation, and neurodevelopment (**Figure 7-1**).

7.1.1 Synthesis

225. All vertebrates possess the vitamin D endocrine axis.[574] Aquatic vertebrates obtain vitamin D solely from the diet, while terrestrial vertebrates can obtain vitamin D from both the diet and from the photolytic conversion of 7-dehydrocholesterol to pre-vitamin D_3 in the skin. 7-dehydrocholesterol is present in large quantities in the skin of higher vertebrates and is a precursor molecule in the cholesterol biosynthesis pathway.[569; 575] 7-dehydrocholesterol absorbs UVB light in the 290–315 nm wavelength, which breaks the bond between carbons 9 and 10, creating pre-vitamin D_3. Pre-vitamin D_3 is thermodynamically unstable and rapidly isomerizes to vitamin D_3. [568; 576] This photochemical reaction does not involve any enzymes and is related to the amount of UVB exposure an individual receives. Factors such as latitude, sunscreen use, ethnicity, age, and nutritional status can affect vitamin D_3 production in the skin.[577] Vitamin D_3 is not biologically active and must be metabolized to its active form through two hydroxylation reactions.

226. The first hydroxylation reaction takes place in the liver. Vitamin D_3 is transported from the skin bound to transport proteins in the bloodstream. Most vitamin D_3 is bound to vitamin D binding protein (VDBP), but some is also bound to albumin.[568; 578] Once in the liver, the P450 enzyme 25-hydroxylase (CYP2R1) adds a hydroxyl group to carbon 25, creating 25-hydroxyvitamin D_3. Several P450 enzymes have been shown to hydroxylate vitamin D on carbon 25, but only CYP2R1 is highly expressed in the liver, and mutations in CYP2R1 are linked to low vitamin D levels and rickets. [568; 579-581] Mutations in other candidate P450s do not alter 25-hydroxyvitamin D_3 levels. This hydroxylation step is not well regulated and is dependent on vitamin D_3 substrate availability. Because this reaction reflects the vitamin D_3 status of an individual, measuring serum levels of 25-hydroxyvitamin D_3 is a common method of determining the vitamin D status of patients.[568]

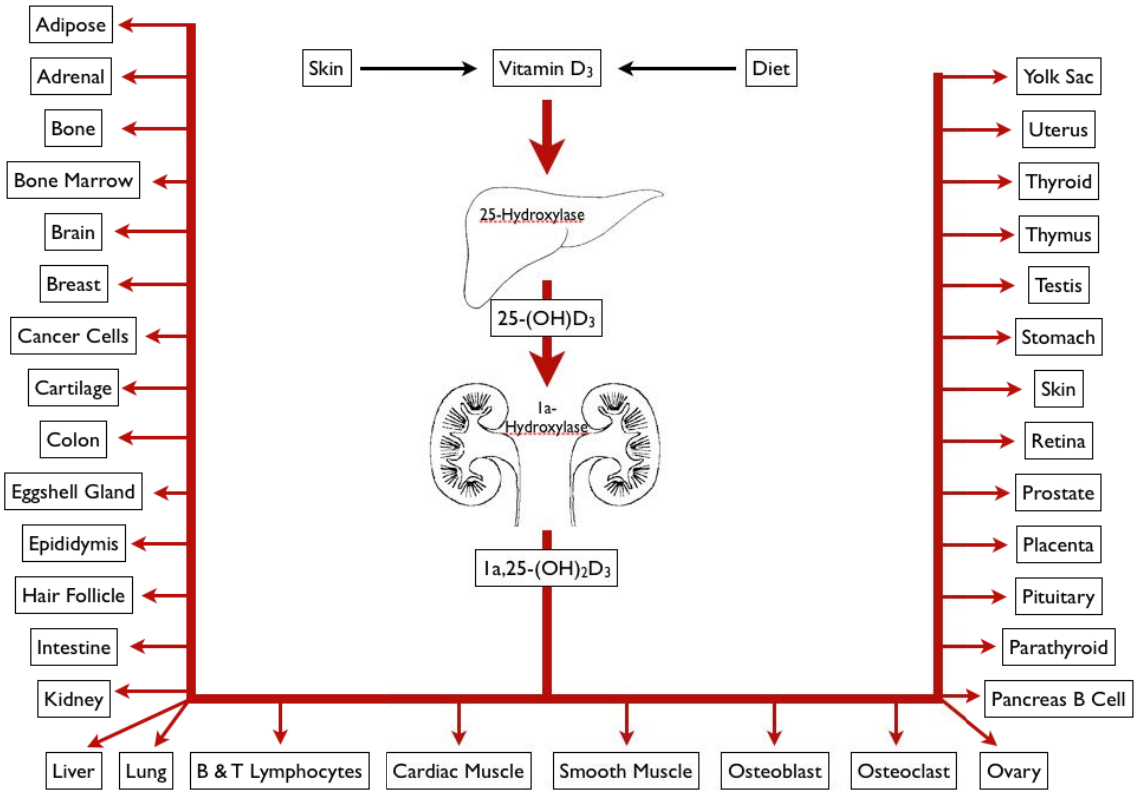

Figure 7-1. Vitamin D synthesis and sites of action.

227. After the initial hydroxylation, 25-hydroxyvitamin D_3 is once again bound to transport proteins and transported in the blood from the liver to the kidney for the second hydroxylation reaction. The 25-hydroxyvitamin D_3-VDBP complex is filtered out of the blood by the glomerulus and is absorbed at the proximal tubules of the kidney by endocytosis mediated by a surface receptor protein called megalin.[582] Megalin-deficient mice are unable to reabsorb 25-hydroxyvitamin D_3 at the proximal tubules and instead excrete the vitamin D_3 metabolite in their urine. These mice suffer from vitamin D deficiency and rickets.[582] Once inside the cells of the proximal tubules,VDBP is degraded while 25-hydroxyvitamin D_3 is transported to the mitochondria for the second hydroxylation. The P450 enzyme 1α-hydroxylase (CYP27B1) adds a hydroxyl group to carbon 1 of 25-hydroxyvitamin D_3, creating 1α, 25-dihydroxyvitamin D_3, which is the active metabolite of vitamin D_3.[568]

228. Unlike the first hydroxylation, the second hydroxylation is tightly regulated. This regulation is necessary in order to maintain proper 1α, 25-dihydroxyvitamin D_3 levels. One of the major roles for vitamin D in vertebrates is calcium homeostasis through the control of calcium absorption in the intestine, and releasing calcium from skeletal stores. Calcium-sensing receptors in the parathyroid gland detect when serum calcium levels are low and trigger the release of parathyroid hormone (PTH). PTH induces the expression of 1α-hydroxylase, which increases the concentration of 1α, 25-dihydroxyvitamin D_3, which, in turn, causes an increase in serum calcium concentration.[580; 583] 1α, 25-dihydroxyvitamin D_3 does not directly bind calcium, but binds to its nuclear receptor, the vitamin D receptor, activating it and initiating the transcription genes involved in calcium uptake and transport.

7.1.2 Catabolism

229. 1α, 25-dihydroxyvitamin D_3 regulates its levels by suppressing the expression of CYP27B1 and by inducing the expression of its major catabolism enzyme: 24-hydroxylase (CYP24A1). This P450 enzyme initiates the breakdown of 1α, 25-dihydroxyvitamin D_3 to calcitroic acid through a series of hydroxylations and side chain oxidations.[581] The breakdown products are eliminated from the body. Nearly all cells in the body express 24-hydroxylase, but the highest activity is in the kidney.[583] Mice lacking CYP24A1 cannot clear 1α, 25-dihydroxyvitamin D_3 from their bloodstream, and the active form of vitamin D remains in their bloodstream for days.[584] Many other compounds and receptors are capable of inducing CYP24A1 expression, suggesting that these compounds could have an effect on the vitamin D status of the animal. 24-hydroxylase is regulated by many of the same compounds as 1α-hydroxylase, but in an opposite fashion.

7.1.3 Calcium and Skeletal Maintenance

230. Classically, vitamin D is necessary for normal bone development and remodeling. Vitamin D-VDR signaling controls the differentiation of bone-forming osteoblasts and bone-resorbing osteoclasts. The vitamin D controlled balance between these two cell types is necessary for proper bone growth and function.[585] Vitamin D regulates many actions of osteoblasts, including cell proliferation, bone matrix synthesis, mineralization, and the initiation of osteoclastogenesis.[586; 587]

231. Vitamin D and VDR are both necessary for the expression of transport channels and proteins necessary for proper calcium absorption from the small intestine.[568] Vitamin D and VDR are also necessary for proper skeletal growth in the young and skeletal maintenance in adults. Vitamin D deficiencies result in the bone-softening disease, rickets, in the young and in osteomalacia in adults.

7.1.4 Immune System Function

232. VDR is widely expressed in multiple immune cell types, including testosterone lymphocytes, macrophages, and dendritic cells.[588; 589] Immune cells are capable of producing and maintaining local concentrations of 1α, 25-dihydroxyvitamin D_3 through the expression of both 1α-hydroxylase and 24-hydroxylase (reviewed in Bouillon et al., 2008[575]). 1α, 25-Dihydroxyvitamin D_3 is thought to play a role in the differentiation and function of immune cells. The lack of vitamin D contributes to the etiology of multiple autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes.[586; 590-592] Vitamin D prevents or suppresses autoimmune diseases by preventing the immune system from attacking body tissues, and proper vitamin D supplementation during infancy and childhood has been shown to decrease the incidence of autoimmune disease in adult life.[589]

233. Vitamin D is either synthesized in the skin or obtained through the diet. Vitamin D_3 is transported to the liver and undergoes the first hydroxylation reaction by 25-hydroxylase, creating 25-hydroxyvitamin D_3. This compound is transported to the kidneys for the second hydroxylation by 1α-hydroxylase to create the active metabolite: 1α, 25-hydroxyvitamin D_3. The active form is carried in the blood to multiple tissues in the body, where its biological functions are mediated though binding to and activating the VDR. The list of tissues for this diagram was taken from Table 1 in Norman.[569]

7.1.5 Cancer

234. VDR and vitamin D status have an inverse relationship with the incidence of multiple cancers, including breast, colon, and prostate cancers. Additionally, there is an inverse relationship between many cancers and UVB exposure.[586] The activation of VDR by vitamin D in cancer cells has been shown to inhibit cancer cell proliferation, induce apoptosis, inhibit angiogenesis, and decrease the metastatic

potential of cancer cells. Vitamin D analogs are currently being studied as potential therapeutic agents in cancer treatment.[568; 586]

7.1.6 Neurodevelopment

235. The vitamin D receptor and P450 enzymes involved in vitamin D synthesis and catabolism are expressed in the brain, CNS, and PNS.[568; 586] Vitamin D is an important neurosteroid, with critical roles in vertebrate brain development.[593; 594] Numerous studies have shown that gestational vitamin D deficiency results in offspring with abnormal brain development. Developmental alterations in mouse models include abnormal brain size, increased cell proliferation, decreased cortical brain thickness, and altered neurotransmitter production.[593; 594] The effects of developmental vitamin D deficiency are often permanent in adulthood.

236. Vitamin D activates both tyrosine hydroxylase and choline acetyltransferase, which are important for the production of dopamine, noradrenaline, adrenaline, and acetylcholine. These neurotransmitters are known to have roles in neurobehavioral disorders such as autism, schizophrenia, and ADHD. Vitamin D deficiency has been linked to an increased risk for these disorders. Many risk factors for vitamin D deficiency, such as living in areas with little UV light exposure, are also linked to increased risk for schizophrenia, autism, and other mental health disorders.[593; 594]

237. Although vitamin D deficiencies can result in neurodevelopmental disorders, adequate levels of vitamin D may have neuroprotective effects. For example, vitamin D increases levels of nerve growth factor (NGF), which is believed to counteract neural degeneration in Alzheimer's disease.[595] Vitamin D also helps defend the brain against oxidative degeneration by increasing the expression of γ-glutamyltranspeptidase. This enzyme is involved in the production of the antioxidant glutathione.[593] Vitamin D has also been shown to protect against the neurotoxic effects of the street drug methamphetamine.[596]

7.1.7 Cardiac Function

238. Cardiac disease is the most common cause of mortality and morbidity in the United States. Many cardiovascular cells express VDR and respond to 1α, 25-dihydroxyvitamin D_3. One such system is the renin-angiotensin system directly regulates blood pressure and electrolyte homeostasis. Renin is a protease that cleaves angiotensin I from angiotensinogen. Angiotensin I is converted to angiotensin II, which exerts its effects on multiple organs to regulate blood pressure and electrolyte balance. The production of angiotensin II is tightly regulated, and the overproduction of angiotensin II has been linked to hypertension, heart attack, and stroke.[586] 1α, 25-dihydroxyvitamin D_3-bound VDR directly inhibits renin expression by binding to the VDRE in the promoter of the renin gene. In VDR-null mice, renin expression was increased, leading to hypertension, cardiac hypertrophy, and increased water intake. Vitamin D supplementation was shown to significantly decrease blood pressure in multiple human studies.[597] There is a strong correlation between vitamin D deficiency and many cardiovascular diseases, including hypertension, coronary artery disease, and heart failure.[597]

7.1.8 Metabolism of Secondary Bile Acids

239. Bile acids are end products of cholesterol metabolism that play an important role in the intestinal absorption of lipids.[598] Bile acids aid in lipid digestion by breaking up large lipids into smaller droplets, and aid lipid absorption by forming water-soluble micelles around the droplets. Bile acids are produced in the liver and secreted into the duodenum. Bile acids are stored in the gallbladder between meals. After lipids are digested and absorbed, bile acids are returned to the liver through enterohepatic circulation.

240. Not all bile acids are recycled. In the large intestine, bile acids may undergo bacterial dehydroxylation, forming a new compound. These end products are called "secondary" bile acids, in reference to the role of the bacterial modification in their creation. "Primary" bile acids refer to bile acids that are synthesized from cholesterol in the hepatocyte of the liver.[599] The most common secondary bile acid in humans is lithocholic acid (LCA). LCA is formed from the primary bile acid chenodeoxycholic acid (CDCA). Unlike CDCA, LCA is a highly toxic and carcinogenic compound and has been shown to cause DNA strand breaks, form DNA adducts,and inhibit DNA repair enzymes, and has been linked to colon cancer.[600] There is a positive correlation among high-fat diets, increased LCA concentrations, and colon cancer.

241. LCA is broken down in the intestine by the P450 enzyme CYP3A4, which is also under the regulatory control of vitamin D, suggesting that vitamin D has an important role in LCA detoxification. LCA and its major metabolites have been shown to be VDR ligands, binding to and activating VDR and inducing the expression of CYP3A4.[601; 602] Other bile acid receptors such as FXR and PXR can be activated by LCA, but VDR is activated at much lower concentrations. Vitamin D increases CYP3A4 expression, thus decreasing the levels of LCA. Indeed, vitamin D and calcium levels are related to reduced incidence of colon cancer, and vitamin D supplementation reduces colon cancer risk.[600] VDR-mediated protection against colon cancer is decreased in situations, resulting in vitamin D deficiency or in high-fat diets. The highest death rates from colon cancer occur in areas with a high prevalence of rickets.[600]

242. It should be noted that vertebrate bile acid and alcohol evolution is extremely complex, with many vertebrates having bile acids or alcohols that are unique to that species. For a detailed, in-depth review of vertebrate bile acids and alcohols, see Hagey et al.[598] and Hofmann et al.[603]

7.2 Consequences of Disruption

243. Most of the current knowledge of the consequences of vitamin D signaling pathway disruption has been gained through the use of knock-out mouse models and, to a lesser extent, studying vitamin D-related diseases in humans. Few studies have addressed vitamin D signaling disruption in wildlife. VDR knock-out mice are born phenotypically normal, but show decreased levels of calcium absorption after weaning. The decreased serum calcium levels lead to hypocalcemia, hyperparathyroidism, and elevated serum 1α, 25-dihydroxyvitamin D_3 levels. These animals develop severe growth retardation, rickets, and osteomalacia. Bones of VDR knock-out mice are more fragile compared to their wild type counterparts due to decreased bone mineralization and the uncoupling of bone remodeling. VDR is necessary for proper calcium absorption, and the lack of calcium absorption in the VDR knock-out is thought to be responsible for the skeletal phenotype seen. This phenotype can be "rescued" with a high calcium diet, supporting the hypothesis of decreased calcium absorption as the cause for the skeletal phenotype. 1α-hydroxylase knock-out mice show a similar skeletal phenotype, although these mice have undetectable levels of 1α, 25-dihydroxyvitamin D_3 and elevated levels of 25-hydroxyvitamin D_3. The skeletal phenotype of the 1α-hydroxylase knock-out can be rescued with the administration of 1α, 25-dihyroxyvitamin D_3 and a high calcium diet.[568; 575; 604] In humans, vitamin D–dependent rickets type I (VDDR-I) is associated with the loss of 1α-hydroxylase, and vitamin D–dependent rickets type II (VDDR-II) is associated with the loss of VDR. The knock-out mouse models for 1α-hydroxylase and VDR are both used as animal models of human disease.

244. The role of vitamin D extends beyond the skeleton. Knock-out mouse models have shown that the disruption of the vitamin D endocrine pathway can have detrimental impacts on additional vitamin D target systems. As described above in Section 7.1.6, *Neurodevelopment*, vitamin D is important for vertebrate neural development. VDR knock-out mice display abnormal muscle and motor behavior and abnormal cognition.[575] Numerous studies have shown that gestational vitamin D deficiency results in

offspring with abnormal brain development. Developmental alterations in mouse models include abnormal brain size, increased cell proliferation, decreased cortical brain thickness, and altered neurotransmitter production.[593; 594] The effects of developmental vitamin D deficiency on the nervous system are often permanent in adulthood.

245. VDR is widely expressed in the immune system and is necessary for proper immune system function. Impaired immune defense has been linked to vitamin D deficiency.[568] The loss of vitamin D is also linked to an increased risk for multiple autoimmune diseases. The loss of vitamin D and VDR also have been linked to increased risk for heart disease and many types of cancer.[575]

7.3 Precedent Chemicals

7.3.1 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

246. The VDR maintains high substrate fidelity; thus, few EDCs are likely to interact with this receptor directly.[571] However, studies in laboratory animals and wildlife exposed to dioxins (such as TCDD) and dioxin-like compounds have shown altered vitamin D_3 serum levels and associated bone malformations.[605-609] Although this evidence suggests that dioxins may be disrupting the vitamin D endocrine system, it is fairly clear that dioxins are not VDR ligands.[573] Dioxins are ubiquitous and persistent environmental contaminants and potent endocrine disruptors in multiple biological systems. The effects of dioxin exposure include reduced reproductive success, decreased survival of early life stages, and perturbations in growth and development.[610] Classic signs of TCDD toxicity in teleosts include alterations in cardiovascular development and function, craniofacial malformations, delayed growth, and death.[610; 611] Effects of TCDD are mediated by the AhR in vertebrates. Although the endogenous ligand and role for AhR are unknown, AhR has an important role in the metabolism of many xenobiotics.[612] Xenobiotic detoxification is the classic role of AhR. It is also thought to be associated with organogenesis and development.[612; 613] Like VDR, AhR is expressed in both osteoblasts and osteoclasts.[614; 615] TCDD has been shown to inhibit osteoblast differentiation and osteoclastogenesis, but the mechanism(s) of action remain unknown.[614; 616] Vitamin D 1-hydroxylase and 24-hydroxylase, the two most important P450 enzymes for maintaining vitamin D homeostasis, have recently been shown to be AhR targets.[607; 617]

247. The current understanding of putative association(s) between TCDD, vitamin D, and the resulting effects on bone development and remodeling is poor. Few studies have evaluated the effects of TCDD on bone formation, and even fewer studies have included the assessment of a putative vitamin D mechanism. One study found that mouse pups that were lactationally exposed to TCDD had increased expression levels of 1-hydroxylase and increased levels of serum vitamin D.[608] Lake Ontario is highly contaminated with dioxins, and mink consuming fish from the lake have a specific jaw lesion that is considered a biomarker for TCDD exposure.[606] While there are likely several mechanistic links between AhR activation and bone/cartilage modifications, few studies have examined AhR modifications within the vitamin D endocrine axis.

7.3.2 Polychlorinated Biphenyls (PCBs)

248. Polychlorinated biphenyls (PCBs) are persistent organic pollutants that were commonly used as coolants and insulators in capacitors and transformers. Exposure to PCBs has been shown to alter bone homeostasis, strength, and composition. Few studies exist that assess PCB effects on vitamin D_3. Alvarez-Lloret et al.[609] found decreased serum vitamin D_3 levels and decreased bone mineralization and composition in rats exposed to PCB126. Lilienthal et al.[608] also noted decreased serum vitamin D_3 levels in rats exposed to a PCB mixture. Wild seals exposed to high PCB and DDT levels exhibited bone lesions that may have been related to a disruption of the vitamin D and thyroid hormone pathways.[605] The exact

mechanism of action of PCB disruption of the vitamin D endocrine pathway is unknown, but may involve AhR activation.

7.3.3 Ethanol

249. Chronic alcohol consumption can alter bone growth and remodeling, resulting in decreased bone density and an increased risk of bone fractures.[618] Studies in rats have shown that chronic alcohol consumption results in reduced serum 1α, 25-dihydroxyvitamin D_3 levels as a result of both decreased CYP27B1 and increased CYP24A1 expression.[619] Other studies have shown similar results in rats and chickens.[620; 621]

7.3.4 Lead

250. Many VDR polymorphisms exist in the human population. Some are associated with decreased bone density, hyperparathyroidism, resistance to vitamin D, and increased susceptibility to infections, autoimmune diseases, and cancers.[568] Three VDR polymorphisms— *BsmI, ApaI, FokI* (named for their identifying restriction sites)—have been shown to affect lead concentrations in whole blood and plasma.[622] Lead accumulates in bone tissue during bone growth and remodeling and has been shown to compete with calcium for common transport mechanisms.[623] During normal bone remodeling, stored lead is released into the bloodstream. Individuals who are homozygous for these polymorphisms have lower concentrations of lead in their whole blood and plasma.[622] It is thought that these polymorphisms produce a less-functional VDR, which could potentially reduce lead accumulation in the bone, leading to lower blood and plasma concentrations from bone remodeling. More studies are needed to better understand the functionality of these polymorphisms. Lead is teratogenic to the developing fetus, and one study has suggested that the presence of all three of these polymorphisms may have a protective effect on the fetus against lead exposure.[624] Although these VDR polymorphisms may have a protective effect against lead toxicity, it is important to remember that these VDR polymorphisms may be less functional and could cause other health consequences related to the vitamin D endocrine system.

7.4 In Vitro Assays

251. There are very few studies that have evaluated the effects of contaminants on the vitamin D signaling pathway. Of those conducted, specific endpoints incorporated include assessment of contaminants on serum vitamin D levels, vitamin D receptor binding, transcriptional activation of vitamin D target genes, and assessment of apical endpoints within the vitamin D endocrine axis, including alterations in skeletal morphology. Common methods used in these studies are summarized below.

7.4.1 Transactivation VDR Reporter Assays

252. Transactivation reporter assays, as described in previous sections, have been used to assess the responsiveness of VDRs from different species or different isoforms from the same species to vitamin D.[573; 625; 626] However, we are aware of no reports of the use of a VDR reporter assay to evaluate responsiveness to potential EDCs. The high fidelity of the VDR may severely limit the promiscuity of the receptor. Transactivation reporter assays would be a viable means of assessing chemical interactions with the VDR when structure-activity analysis or apical toxicity suggests such a molecular interaction.

7.4.2 AhR Transactivation Reporter Assays

253. Considering that AhR agonists can modulate vitamin D levels by inducing enzymes involved in vitamin D metabolism (see Section 7.3.1, *2,3,7,8-tetrachlorodibenzo-p-dioxin*), AhR transactivation

assays would facilitate the identification of putative disruptors of vitamin D signaling. AhR transactivation reporter assays are described in Section 5.4.1, *AhR Transactivation Reporter Assay*.

7.5 In Vivo Assays

7.5.1 Serum Vitamin D Levels

254. Analyses of circulating vitamin D_3 and vitamin D_3 metabolite levels in exposed and non-exposed populations, or in the same animal both before and after exposure to a chemical, are commonly used to assess the vitamin D endocrine axis. As described above, levels of the active metabolite of vitamin D are maintained through tightly regulated feedback mechanisms governing both its synthesis and catabolism. Other vitamin D metabolites, such as 25-hydroxyvitamin D_3, are less regulated. The conversion of pre-vitamin D_3 to 25-hydroxyvitamin D_3 relies on substrate availability and thus reflects the vitamin D status of an animal. Because serum levels of 1α, 25-dihydroxyvitamin D_3 change depending on the vitamin D needs of the animal, clinicians often measure 25-hydroxyvitamin D_3 to determine vitamin D status. A similar assay to the radioimmunoassay is the enzyme immunoassay (EIA). The EIA offers the advantage of not requiring the use of radioactive material.[627] HPLC and LC-MS/MS also are used to measure serum vitamin D levels; however, these approaches have typically not been used in evaluations of chemical-induced endocrine disruption. RIA is by far the most common method used to assess chemical effects on vitamin D levels. See Wallace et al.[627] for a thorough review of methods and commercial assays.

255. RIA and EIA assays have been successfully used to determine serum vitamin D levels in both laboratory animals and wildlife. Routti et al.[605] used radioimmunoassay to determine circulating levels of 1α, 25-dihydroxyvitamin D_3 in seals exposed to DDT and PCBs and found decreased levels of 1α, 25-dihydroxyvitamin D_3 in exposed populations. Shankar et al.[619] used this approach to measure both serum 25-hydroxyvitamin D_3 and 1α, 25-dihydroxyvitamin D_3 levels in rats after long term ethanol exposure. They observed that ethanol decreased 1α-hydroxylase expression and increased 24-hydroxylase expression, resulting in reduced levels of 1α, 25-dihydroxyvitamin D_3. Levels of 25-hydroxyvitamin D_3 remained unchanged. Nishimura et al.[607] employed an enzyme immunoassay to determine serum 1α, 25-dihydroxyvitamin D_3 levels in neonatal mice that were lactationally exposed to TCDD. They found that exposure to TCDD caused an increase in serum 1α, 25-dihydroxyvitamin D_3 levels as a result of increased 1α-hydroxylase expression.

7.5.2 Microarrays

256. Microarrays have been used previously to unravel the molecular pathway involved in vitamin D signaling, as well as the effects of vitamin D on various target tissue and cancers.[575] To date, microarrays have not been used to study toxicant-induced gene expression changes within the vitamin D endocrine pathway. Microarrays could be a very useful tool to study the effects of a toxicant on the genes involved in vitamin D signaling. Microarrays could also be used to discover previously unknown vitamin D–related genes that are targets of toxicants of concern.

7.5.3 Skeletal Morphology and Bone Densitometry

257. Mineral ion homeostasis within bone is a classical VDR responsive target, and while vitamin D endocrine system has an effect on numerous tissue types, most studies have focused upon the development of skeletal abnormalities in response to chemical disruption. Skeletal abnormalities accompanied by changes in vitamin D status are typically evaluated by measuring changes in various bone characteristics, such as bone mineral density, bone mineral content, bone thickness, mechanical strength, changes in cell content, and gross changes in skeletal structure. Although these measurements

are quite useful when looking at changes in bone morphology, they require specialized equipment that is not available in most laboratories.

258. Nishimura et al.[607] and Finnilä et al.[628] examined the tibias of mice exposed to TCDD for changes in bone characteristics, while Alvarez-Lloret et al.[609] used lumbar vertebra in PCB126 exposed Sprague-Dawley rats. All three groups measured bone mineral density, bone mineral content, and bone thickness, but Finnilä's group also measured the cross-sectional area. Nishimura's group made their measurements using dual energy X-ray absorptiometric analysis, while the Finnilä group and Alvarez-Lloret used peripheral quantitative computed tomography (pQCT). All three groups found that exposure to TCDD or PCB126 caused a significant decrease in bone mineral density, bone thickness, and bone mineral content. Nishimura et al. and Alvarez-Lloret et al. reported decreased vitamin D levels in the treated mice. Finnilä et al.[628] did not measure vitamin D levels.

7.5.4 Histology

259. Histological approaches have been employed to assess both cellular and gross morphological changes following exposure to compounds that may target the vitamin D axis. In general, target tissues are fixed, dehydrated, embedded, and sectioned onto slides. After the tissues are mounted, a wide variety of stains can be used to help visualize the cells or tissues of interest. For example, many contaminants, such as TCDD and PCBs, have been shown to alter bone development. A number of stains can be used to visualize these alterations: Alizarin red S and alcian blue to differentiate between calcified structures and cartilage, alkaline phosphatase to stain bone-forming osteoblasts, tartrate-resistant acid phosphatase (TRAP) to stain bone-resorbing osteoclasts, and Villanueva's Goldner stain to differentiate between mineralized and unmineralized bone.

260. Histology is a common method used in many studies that have examined the effects of contaminants on bone. Nishimura et al.[607] stained tibia sections from 21-day-old TCDD-exposed mice with Villanueva's Goldner stain to distinguish between mineralized and unmineralized bone. These slides were used in morphometric bone analysis. They also used a TRAP staining method to stain for osteoclasts to determine if TCDD induced osteoclastic bone formation. Immunohistochemical approaches can also been used to infer modification of protein expression of target genes following disruption within the vitamin D signaling axis. For example, Nishimura et al.[607] examined calbindin-D28K and 1α-hydroxylase proteins in the kidneys of mice exposed to TCDD and visualized calbindin-D28K in the small intestine and PTH in the parathyroid gland.

7.6 Current Challenges and Limitations

7.6.1 Limited Knowledge Regarding Non-mammalian Vertebrates

261. Most of our knowledge regarding the vitamin D endocrine system has come from mammalian studies. Studies performed with non-mammalian vertebrates suggest that the vitamin D signaling pathway may have important differences among taxa. For example, teleost fish have two copies of the VDR as a result of a whole-genome duplication event specific to the teleost lineage. Mammals and other vertebrates only have one copy.[626; 629] The fact that VDR has been cloned from the sea lamprey (*Petromyzon marinus*) and the little skate (*Leucoraja erinacea*)—two vertebrates lacking a calcified skeleton—suggests that + teleost VDR paralogs have different sensitivities to vitamin D. Additional studies are needed to fully elucidate the functions of early vertebrate VDR and its role in the vitamin D endocrine system.

7.6.2 Broaden Focus beyond Skeletal Effects

262. While it is well known that vitamin D plays a role in numerous systems, tissues, and disease processes, the majority of studies addressing chemical-induced disruption of vitamin D signaling have focused exclusively on bone. Changes in vitamin D levels have been shown to affect many other biological processes, such as development, immune function, nervous system development and function, and disease status (see above); however, apical consequences to these processes resulting from chemical disruption of vitamin D signaling remains largely unknown. The evaluation of additional vitamin D target tissues and systems could broaden our understanding of both the importance of vitamin D signaling and the multi-faceted effects of contaminant exposure (**Table 7-1**).

Table 7-1. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework with most promising assays to detect and characterize chemical effects on the vitamin D signaling pathway*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: Activation/inhibition of VDR; Activation of AhR	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	VDR transactivation reporter assay; AhR transactivation reporter assay
Tissue-level responses Induction of vitamin D metabolizing enzymes	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	Vitamin D.hydroxylase assay, EROD activity assay (biomarker) (could potentially be applied to any *in vivo* exposure assays)
Organ-level responses Reduced serum vitamin D levels	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	RIA/EIA for serum vitamin D levels (could potentially be applied to any *in vivo* exposure assays)
Whole organism responses Neuro-developmental abnormalities; reduced skeletal growth	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Brain size measurements in rodent offspring; reduced bone length in juvenile rodent (assays have been performed in mice, could potentially be applied to rat 2-generation assays) (TG 416, TG 443)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR SIGNALING PATHWAY

8.1 Overview

263. PPARs are type II nuclear receptors; therefore, they are typically localized to the nucleus, unlike the type I receptors that translocate to the nucleus following ligand binding in the cytoplasm. There are three distinct PPARs in mammals—PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3)—and all three PPARs heterodimerize with the RXR to initiate their transcriptional actions.[630] Similar to most nuclear receptors, PPARs have five domains, designated A/B, C, D, E, and F (see Section 1, *Introduction*, for a discussion of nuclear receptor domains). The A/B domain of each receptor is poorly conserved[631] and, in part, this domain regulates the transcription of specific target genes by each PPAR isoform.[632] The A/B domain also contains the AF-1 region, which has low level basal activity and the crucial phosphorylation sites for the ligand-independent transcriptional activation of PPARα[633; 634] and repression of transcriptional activity by PPARγ.[635; 636] The C domain, or DBD, is highly conserved among the three receptors and activates transcription primarily at different DR-1 response elements.[637] The D domain is a hinge region that links the DNA and LBDs, but also contains co-repressor binding sites.[638] The LBD encompasses the E/F domain, which houses AF-2 for interaction with co-activators, such as PGC-1α, SRC-1, and CBP/p300.[638; 639] **Table 8-1** lists several of the functions of each PPAR.

264. The LBD of the PPARs is large, with a binding pocket of approximately 1300–1400 Å, and can accommodate large ligands such as fatty acids. The LBD for PPARα and PPARγ are quite similar and show affinity for similar ligands. For example, a one amino acid difference accounts for the increased pharmacological sensitivity of PPARγ for the thiazolidinedione drugs[640] and the greater lipophilicity of the binding pocket of PPARα may account for its higher affinity for saturated fatty acids. In contrast, PPARβ/δ has a large LBD, but its pocket is much more narrow.[640]

265. PPARs are activated by fatty acids, pharmacological ligands, and other xenobiotics, and, in turn, regulate genes involved in fatty acid metabolism, inflammation, and proliferation. Each of the PPARs shows different tissue expression and functions. PPARα is primarily expressed in the liver, intestine, kidney, heart, and adipose tissue.[631; 638] PPARα controls β-oxidation in the peroxisomes and mitochondria and ω-oxidation in the endoplasmic reticulum of the liver. In turn, PPAR ligands reduce VLDL (very low density lipid), increase HDL (high density lipid), and reduce the duration of macrophage-induced inflammation.[641; 642] PPARβ/δ is ubiquitously expressed, but intestinal epithelium, liver, and keratinocytes account for its highest expression, consistent with data indicating that PPARβ/δ activation improves glucose tolerance and mediates cellular differentiation of skin and intestine.[643] PPARβ/δ activation also improves fatty acid catabolism in skeletal muscle.[644; 645] There are three isoforms of the PPARγ gene—PPARγ1, PPARγ2, and PPARγ3. PPARγ1 is expressed across a wide variety of tissues, although at low levels. PPARγ2 and PPARγ3 are expressed in adipose, and PPARγ3 is also expressed in macrophages. Here, PPARγ regulates adipocyte differentiation and represses inflammation.[646]

Table 8-1. General function of each of the PPARs in vertebrates.

PPAR	Involvement
PPARα	Facilitates peroxisome proliferation, liver cancer, fatty acid metabolism, and developmental delay. Alters lipid homeostasis, inhibits inflammation,
PPARβ/δ	Increases fatty acid metabolism. Facilitates skin proliferation and differentiation. Facilitates placental development.
PPARγ	Facilitates adipocyte differentiation, glucose homeostasis; controls trophoblast invasion and placental angiogenesis. Represses inflammation.

8.1.1 PPARs in Non-Mammalian Species

266. Most research on PPARs has been conducted with mammals. Information provided on PPARs in this section has largely been determined in mammals. However, PPARs also have been detected in chicken, *Xenopus*, and several fish species. The fact that peroxisome proliferation is mediated through PPARs was first discovered in *Xenopus*,[647] and three PPARs have been identified in *Xenopus*, PPARα, PPARβ/δ, and PPARγ. [637] The chicken genome also contains all three PPAR members, with similar expression profiles as mammals.[648; 649] Studies indicate the presence of four PPARs in the Japanese pufferfish *(Fugu*) genome: two PPARαs, PPARδ, and PPARγ.[650] Interestingly, zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) express two distinct PPARβ/δ genes.[651; 652] The four PPARs in *Fugu* show wide tissue distribution, whereas in mammals, only PPARβ/δ is widely distributed. Sea bream (*Sparus aurata*), plaice (*Pleuronectes platessa*),[651] and sea bass (*Dicentrarchus labrax*) also express PPARα, PPARβ/δ, and PPARγ. However, these studies indicate that PPARα and PPAR β/δ demonstrate similar tissue distribution to mammals, while PPAR β/γ showed wide tissue distribution. Using mouse antibodies, PPARα, β, and γ have also been detected in the liver of gray mullet (*Mugil cephalus*) and zebrafish, which may make fish sensitive to the effects of peroxisomal proliferators.[653; 654]

8.2 Consequences of Disruption

267. Obesity has increased at an alarming rate. Adult obesity has more than doubled since 1980 and, as a consequence, one-third of adults in the United States have a body mass index (BMI) greater than 30 and are considered obese, and more than two-thirds of the adult population in the United States have a BMI greater than 25, and therefore are considered overweight.[655] The rate of obesity in the United States is approximately 2 times greater than the rest of the world; however, several other nations have significant obesity problems, including but not limited to Bahrain (29% are obese), American Samoa (75%), Panama (35%), Mexico (24%), the United Kingdom (23%), the United Arab Emirates (34%), Nauru (79%), Kiribati (51%), Israel (23%), Greece (23%), and Chili (22%).[656] Approximately 200,000,000 men and 300,000,000 women worldwide are obese,[656] and obesity rates are growing rapidly in parts of Europe, Latin America, the Caribbean, and the Middle East.[657; 658]

268. As a result, diseases associated with obesity have increased. Common metabolic disorders associated with obesity include insulin resistance, glucose intolerance, hypertension, and dyslipidemia, all of which place an enormous burden on healthcare systems and cause rising healthcare costs. There are many methods to mitigate the problem, including increasing physical activity, reducing food portions, and improving food choices. However, pharmacological interventions and environmental toxicants may exacerbate these conditions. PPARs, which regulate lipid and energy homeostasis, are potential targets for environmental chemicals. In this era where obesity is a worldwide epidemic, any effects on receptors that regulate lipid and energy homeostasis may be critical area for endocrine disruption research.

269. In 2002, Baillie-Hamilton suggested that weight gain may be caused by environmental toxicants. The investigator suggested that weight gain in toxicity studies was being overlooked by toxicologists who were primarily interested in weight loss as a symptom of toxicity.[659] A few years later, the term "obesogen" was coined.[7] Another term used is "metabolic disruptor."[660; 661] Both terms define a new subclass of endocrine disruptors that perturb metabolic signaling and energy (lipid) homeostasis, leading to increased weight, adipogenesis, and obesity in rodent models and perhaps the human population. The primary receptors of interest are the PPARs, with special interest in PPARγ. Below are the basic functions of each PPAR (PPARα, PPARβ/δ, PPARγ), as well as the potential consequences of PPAR disruption.

8.2.1 PPARα

270. PPARα's primary purpose is the regulation of energy homeostasis. PPARα activates fatty acid catabolism (i.e., β-oxidation in the peroxisomes and mitochondria and ω-oxidation through CYP4A in the endoplasmic reticulum), increases gluconeogenesis and ketone body synthesis, controls the production of lipoproteins, and enhances the catabolism and elimination of cholesterol.[638] Additionally, PPARα attenuates inflammatory responses and, consequently, PPARα-null mice have prolonged inflammatory responses.[641]

271. In general, the activation of PPARα in humans seems to have a beneficial effect on health. PPARα activation lowers plasma triglyceride levels and reduces adiposity, which, in turn, improves insulin sensitivity.[662-664] Because of this, PPARα activators such as the fibrate drugs reduce hypertriglyceridemia in humans. PPARα-null mice are unable to respond to fibrate drugs; therefore, their hyperlipidemia does not improve, demonstrating that these effects are PPARα-dependent.[638; 662] PPARα also protects against muscle and hepatic steatosis, including diet-induced steatohepatitis in mice and humans.[664-666] Furthermore, the anti-inflammatory effects of PPARα agonists have positive effects on the cardiovascular system.[667]

272. However, PPARα ligands are peroxisome proliferators, and they promote liver carcinogenesis in rodent models.[668-670] PPARα ligands have not been shown to cause mutations, and thus, are considered nongenotoxic carcinogens. Current hypotheses suggest that PPARα ligands promote cancer because they increase mitochondrial and peroxisomal β-oxidation by inducing medium-chain acyl-CoA dehydrogenase and acyl CoA oxidase.[671; 672] Further, peroxisome proliferators increase hydroxylation of fatty acids by inducing CYP4A family members.[673] This, in turn, increases reactive oxygen species and perturbs eicosanoid homeostasis. These changes may play a role in cell proliferation and carcinogenesis.

273. Nevertheless, there is currently little evidence that PPARα ligands and peroxisome proliferators cause liver cancer in humans, and peroxisome proliferators currently are not considered human carcinogens.[674; 675] PPARα is highly expressed in rodent liver, but weakly expressed in humans, and this is thought to be the underlying cause of most of the species differences in toxicity related to peroxisome proliferation. Evidence that expression is a key regulator of peroxisome proliferation is that adenoviral-driven expression of hPPARα in mice induces peroxisome proliferation.[676] Conversely, PPARα-null mice humanized with the hPPARα gene within an artificial chromosome containing 100 kb of the 5'-regulatory region and 23 kb of the 3'-regulatory region of hPPARα do not develop hepatocyte hyperplasia while still mediating many of the functions ascribed to PPARα, including hepatocyte hypertrophy (Yang et al., 2008). Taken together, peroxisome proliferators are not considered carcinogens in humans.[674; 675]

274. PPARα is also expressed during fetal development,[631] and the fetus may therefore be susceptible to PPARα ligands. For example, both PFOS and PFOA activate PPARα and initiate development defects in mice. PFOA reduced survival, delayed eye opening, and caused decreased body weight;[631] however,

these effects are lost in PPARα-null mice, demonstrating that PPARα mediates the adverse effects of PFOA on development. However, the effects of PFOS are not lost in PPARα-null mice. Overall, there are few published studies indicating PPARα-dependent effects on development; therefore, it is difficult to discern whether developmental defects are a characteristic adverse effect produced by PPARα ligands. In addition, these studies were performed in rodents, and PPARα expression is different in several tissues in rodents compared to humans during gestation.[631] Whether the developmental defects in mice caused by PPARα ligands are relevant to humans is currently unknown.

8.2.2 PPARβ/δ

275. PPARβ/δ controls energy homeostasis by regulating genes involved in fatty acid catabolism and adaptive thermogenesis in the heart, skeletal muscle, liver, and fat.[645; 677] PPARβ/δ is also involved in development and cell proliferation. PPARβ/δ controls cell proliferation, cell migration, differentiation, survival, and tissue repair and is critical in the development of the placenta and digestive tract.[662; 678-680] Lastly, PPARβ/δ has anti-inflammatory properties mediated by macrophages.[645; 662]

276. In animal models, PPARβ/δ agonists reduce weight gain caused by a high-fat diet and, in turn, maintain insulin sensitivity, probably by increasing skeletal muscle fatty acid catabolism and thermogenesis.[681] Whether PPARβ/δ ligands reduce weight gain in humans is not known.[645] One of the most promising aspects of PPARβ/δ activation is the increase in HDL coupled with lower cholesterol and triglycerides.[682] Several therapeutics reduce cholesterol or triglycerides, but few therapeutics positively affect HDL levels.[645] Furthermore, the gain of function VP16-PPARa/b transgenic mice have lower body weights, reduced inguinal fat mass, decreased triglyceride accumulation in their adipocytes, and lower free fatty acids circulating in their blood compared to control littermates. In contrast, PPARβ/δ-null mice are more susceptible to weight gain.[681] Thus, PPARβ/δ has positive actions on triglycerides, cholesterol, HDL, and weight gain in rodent and rhesus monkey models.

277. Skeletal muscle is another area where PPARβ/δ activity is crucial. Skeletal muscle accounts for almost 80% of insulin-stimulated glucose uptake. PPARβ/δ agonists increase the expression of genes involved in fatty acid oxidation, mitochondrial respiration, and oxidative metabolism that enhance slow-twitch contraction.[645; 683; 684] This leads to increased oxidative (fatty acid) metabolism rather than use of the glycolytic pathways involved in muscle respiration. In turn, endurance is increased and, not surprisingly, marathon runners have a high proportion of oxidative fibers compared to glycolytic fibers. In contrast, obesity and insulin resistance are associated with a loss of oxidative fibers.[685] Concurrently, PPARβ/δ ligands increase fatty acid oxidation in the heart, which also primarily uses fatty acids for energy.[686] This increases heart contractile function.[687]

278. Initially, these physiological effects do not appear to be the type of perturbations that should concern regulators; however, there may be unforeseen consequences on human activity, predator avoidance, and food quality if PPARβ/δ metabolic disruptors entered the environment with regularity. Currently, we know less about PPARβ/δ function than the other PPARs. Therefore, it is not surprising that we have fewer pharmacological ligands and few environmentally relevant disruptors of PPARβ/δ function. It is possible that there are few side effects or problems associated with PPARβ/δ disruption. However, there are significant gaps in our knowledge that need to be addressed to determine whether there are PPARβ/δ disruptors and whether their effects are adverse.

8.2.3 PPARγ

279. PPARγ is crucial in adipose tissue differentiation and adipocyte function, such as fat storage and energy dissipation.[637; 646; 677] PPARγ is pivotal in glucose metabolism because it improves insulin sensitivity.[688] Therefore, the PPARγ ligands such as the thiazolidinediones improve insulin sensitivity and reduce hyperglycemia and are useful treatments for type 2 diabetes.[646] Inflammation is also impeded by PPARγ agonists, thus providing additional roles for the zolidinediones in improving atherosclerosis and diabetes.[662]

280. Unlike the other PPAR receptors, PPARγ activity has some clear downsides. Heterozygous PPAR +/- mice show reduced weight gain after treatment with a high-fat diet.[646] High PPARγ activity also is associated with obesity in humans. For example, a mutation (P116Q) in the PPARγ2 isoform decreases MAPK-mediated phosphorylation, leading to increased activity and severe obesity.[689] PPARγ antagonists prevent weight gain in high-fat diet treated rodents.[690-692] In addition, a side effect of the prolonged use of thiazolidinediones is weight gain,[646] but thiazolidinediones are continually used because the benefits of the glitazones outweigh their side effects for persons with type 2 diabetes.

281. Therefore, one might presume that activation of the PPARγ pathway in healthy individuals is probably contra-indicated because of its side effects. This is probably an oversimplification and may not be the case because of perturbations in insulin signaling. For example, individuals with a polymorphism in the N-terminus of the PPARγ2 isoform (P12A) have lower transcriptional activity. This polymorphism is associated with reduced BMI and improved insulin sensitivity in some populations.[646] However, other studies have failed to observe this phenotype or have observed increased BMI, especially in Caucasians or individuals with a BMI greater than 27.[693-695] Therefore, it may be that moderation is key and that over and under-activity may have metabolic consequences.

282. Chemically induced PPARγ activity causes obesity, as determined by studies that demonstrate that glitazones (thiazolidinediones) increase weight gain.[646; 696; 697] In addition, environmentally relevant PPAR agonists increase weight gain and lipid deposition, and a majority of these show activity towards PPARγ,[7; 655; 698-700] with some showing additional PPARα activity.[631; 698; 701; 702] Furthermore, urinary concentrations of phthalate metabolites are associated with increased waist circumference and insulin resistance.[703] The promiscuous nature of some of these chemicals such as the phthalates for multiple nuclear receptors complicates their assessment,[698] but given the association of PPARγ agonists with fat deposition, it is an obvious target of concern. A chemical that has received significant attention in this regard is tributyltin.[7; 655; 699; 700]

283. Interestingly, PPARγ activity and adipocytes differentiation can be activated without binding, and instead with increased PPARγ expression. The environmental estrogen, BPA, increases PPARγ expression, and in turn, alters IGF-1 expression and increases early adipogenesis in rats.[704; 705] Furthermore, it may be involved in promoting adipogenesis in 3T3-L1 cells.[706] Halogenated analogs of BPA also bind *Xenopus*, zebrafish, and human PPARγ with greater halogenation, causing increased PPARγ activity, and lower estrogen receptor activity. In addition, these chemicals, which are found in human serum samples, induced adipocyte differentiation in 3T3-L1 cells, indicating that they are potential obesogens.[707]

284. There are other adverse, non-obesogen effects associated with PPARγ activation. For example, DEHP disrupts testicular function, testosterone synthesis, and causes apoptosis. Evidence suggests that DEHP mediates these effects through activation of PPARγ.[708] PPARγ also is associated with the proliferation and the inhibition of proliferation of certain cancers.[709-712] Whether it improves or exacerbates the outcome is currently debated and may depend on specific conditions, and perhaps, even

the type of cancer.[662] PPAR activators such as rosiglitazone have been shown to increase bone fractures presumably because of PPARγ-mediated perturbations in bone remodeling.[713] PPARγ activation promotes hematopoietic stem cell differentiation into osteoclasts, while inhibiting mesenchymal stem cell differentiation into osteoblasts, and instead promotes adipogenesis in collaboration with ERRα and PGC1β.[714-716] Thus, PPARγ activation increases bone resorption and suppresses bone formation; a two-pronged attack on bone formation. The EDC, tributyltin, has been shown to perturb osteoclast differentiation; however, some research suggests the disruption is through the retinoic acid receptor (RAR).[717] Recently, tributyltin, triphenyltin, dibutyltin, and rosiglitazone were shown to disrupt MSC cells and, in turn, increase adipocyte formation in a PPARγ-mediated fashion, which could significantly perturb bone physiology and reduce bone formation.[718]

8.2.4 PPAR Disruption in Wildlife

285. Several fish species and the frog species *Xenopus* and *Rana* have shown peroxisome proliferation and increased acyl-CoA oxidase activity following exposure to PPARα ligands.[671; 719] Exposure to a diverse set of chemicals, including PAHs, phthalates, alkylphenols, and pesticides, has resulted in acyl-CoA oxidase or peroxisome proliferation in fish.[671] For example, the organochlorines endosulfan and dieldrin and the organophosphate disolfoton caused peroxisome proliferation in rainbow trout and gilthead sea bream.[720; 721] 2,4-D treatment increased peroxisome proliferation in mummichogs.[722]

286. Clofibrate increased peroxisome proliferation, with *Rana esculenta* showing greater sensitivity than *X. laevis*.[719] In addition, Clofibrate and gemfibrozil induce embryonic malabsorption syndrome in zebrafish, resulting in small embryos.[723] This effect was reversible when the drugs were eliminated from the media. A recent study demonstrated that gemfibrozil reduced plasma lipoprotein levels and long-chain n-3 fatty acids in rainbow trout (*Oncorhynchus mykiss*), thereby potentially reducing the nutritional quality of exposed fish. The authors also indicated concern for the ability of the fish to adapt to differing water temperatures and reproduce following migration, considering the widespread presence of fibrates in aquatic environments and the role of fatty acids in these adaptation processes.[724]

287. Exposure to PFOA and PFOS perturbed fatty acid concentrations in salmon. Total polyunsaturated fatty acids (PUFA) and monounsaturated fatty acid (MUFA) were increased; specifically, α-linolenic acid, eicosapentaenoic acid, and arachidonic acid increased after PFOS and PFOA exposure. In contrast, PFOA exposure increased DHA levels, but PFOS decreased DHA levels.[725] Taken together, PPARα activators have measurable effects on fish and amphibian species; however, the adverse outcomes of these exposures are poorly understood.

288. A few studies have addressed the effects of PPARγ agonists on fish or amphibians. TBT promotes adipogenesis in *Xenopus laevis.*[7] TBT (10 and 500 ng/L) also perturbed the expression of energy metabolism genes, especially those involved in glucose metabolism and lipid metabolism in a manner consistent with altered AR and PPARγ activity in grey mullet (*Chelon labrosus*).[726] Furthermore, TBT increased body weight and whole-body lipid content in Chinook salmon (*Oncorhynchus tshawytscha*), consistent with an obesogen response, but other parameters were dissimilar. For example, plasma triglycerides and cholesterol were higher in salmon, but lower in mammals following TBT treatment.[727] Interestingly, tributyltin oxide (TBTO) inhibits PPARα and PPARβ/δ activity.[728] It is interesting to speculate that the agonistic effects of TBT on fish PPARγ activity *in vivo* may be perturbed by TBTO's effects on PPARα and β/δ. Other than TBTO, there are few antagonists of PPARs, with the exception of pharmacological antagonists (**Table 8-2**). In summary, there have been few thorough studies of PPAR agonists on environmentally relevant species; therefore, the potential adverse effects on these species are not understood. Pivotal energy-needing behaviors such as migration or reproduction, and the acclimation

to different environmental stressors such as prey avoidance and temperature change, may be compromised under the presence of PPAR ligands.

Table 8-2. Example PPAR activators in mammals.

Chemical	PPARα	PPARβ/δ	PPARγ
1-palmityl-2-oleolyl-*sn*-glycerol-3-phosphocholine	+		
Saturated fatty acids (C14:0 – C18:0)	+	+	
ω-3 unsaturated fatty acids (C18-22)	+	+	+
ω-6 unsaturated fatty acids (C18-20)	+	+	+
ω-9 unsaturated fatty acids (C16-18)	+	+	
Prostaglandin J2			+
Leukotriene B4	+		
Ceramide		+	
8-hydroxyeicosapentaenoic acid	+		
Phytanic acid	+		
Hypolipedmic drugs			
▪ Clofibric acid	+		
▪ Ciprobific acid	+		
▪ Gemfibrozil	+		
▪ Wy-14,643	+		
▪ Eicosatetraynoic acid	+	+	
▪ Benzafibric acid		+	+
▪ GW501516		+	
Indomethacin			+
Ibuprofen			+
Troglitazone			+
Rosiglitizone			+
Pioglitazone			+
Fatty acyl dehydrogenase inhibitors	+		
Carnitine parlmitoyl transferase 1 inhibitors	+		
Phthalates	+		
Mono-2-ethylhexylpthalate			+
Dichloro and trichloroacetic acid	+		
Tributyltin			+
Halogenated analogs of Bisphenol A			+

* There are few antagonists of PPARs. Typical antagonists are pharmacological ligands such as SR-202, GW9662, JTP-426467, HL005 or biphenol-A-diglicydyl ether (BADGE).[690-692; 712; 729; 730] Tributyltin oxide has recently been shown to block PPARα and PPARβ/δ activity in fish.[728]

8.3 Precedent Chemicals

289. PPARs received their name because they cause proliferation of peroxisomes, i.e., organelles that catabolize long chain fatty acids. Given PPARs' large binding pocket, it is not surprising that PPARs are activated by large fatty acids, such as the unsaturated fatty acids linoleic acid, docosahexaenoic acid, linolenic acid, arachadonic acid, and oleic acid.[637] PPARα is also activated by saturated fatty acids of

approximately 12–18 carbons, with a preference for 14–18 carbon saturated fatty acids, but at a lower affinity compared to the unsaturated fatty acids.[637; 731; 732] Recent evidence indicates that the endogenous PPARα ligand in the liver is 1-palmityl-2-oleolyl-*sn*-glycerol-3-phosphocholine.[733] Other endogenous PPAR ligands include ceramides that indirectly activate PPAR β/δ.[734] Leukotrienes and prostaglandins are also activators of PPARs, where leukotriene B4 activates PPARα but PGJ2 does not.[735] PGJ2 preferentially activates PPAR β/γ[736; 737] (**Table 8-2**).

290. In humans and rodent models, the xenobiotic ligands of PPARs include the hypolipidemic drugs and PPARα activators such as Wy 14,463, clofibrate, ciprofibrate, methylclofenapate, clobuzarit, fenofibrate, and foresafen. Pharmaceuticals that activate PPARγ include specific activators such as the glitazones (thiazolidinediones), rosiglitazone, and troglitazone, and the non-steroidal anti-inflammatory drugs such as ibuprofen and indomethacin, which show significantly lower affinity for PPARγ[631; 731; 732] (**Table 8-2**). Several plant extracts have recently been shown to activate all three PPARs. These include carnosic acid and carnasol found in sage and rosemary, which activate PPARγ.[738] In fact, almost 50% of the plant extracts tested showed activation of PPARγ, and over 25% tested showed activation of PPARα;[738] however, the physiological significance of this activation is not known.

291. Several environmental chemicals also have been shown to bind and activate mammalian PPARs. These include plasticizers, pesticides, and anti-fouling agents. For example, phthalate esters and their metabolites activate PPARs,[739] including PPARα activation by mono-benzylphthalate, mono-butylphthalate, and mono-2-ethylhexylphthalate (MEHP).[740] MEHP and 2-ethylhexanoic acid (EHA), which are metabolites of the commonly used phthalate, di-ethylhexylphthalate (DEHP), activate PPARs, with EHA showing a preference for PPARα and MEHP demonstrating similar activity towards both PPARα and PPARγ.[701] However, given that DEHP and MEHP are lipophilic, it has been hypothesized that PPARγ, which is localized in adipose tissue, may be the most physiologically relevant PPAR target.[698; 701] *In vivo* studies indicate that both PPARα and PPARβ/δ may be involved in DEHP toxicity, but in different organ systems. For example, research with PPARα-null mice demonstrate that DEHP induces peroxisome proliferation in a PPARα-dependent manner; however, renal and testicular toxicity occur in a PPARα-independent manner.[741] More recent studies indicate that the testicular toxicity resulting from DEHP exposure in rodents is mediated through PPARβ/δ.[708]

292. PFOS and PFOA activate PPARα. The developmental defects caused by PFOA are lost in PPARα-null mice,[702] demonstrating that PFOA's developmental effects are mediated by PPARα. However, the developmental defects caused by PFOS are not lost in PPARα-null mice.[742] Therefore, the mechanism by which PFOS causes developmental defects remains unresolved, but is not mediated by PPARα. There is also evidence that PFOA activates PPARγ,[743-745] but some laboratories have not been able to verify this result.[746] Perfluorochemicals also activate PPARα in the Baikal Seal (*Pusa Sibirica*);[747] however, whether this results in peroxisome proliferation is not known.

293. Trichloro- and dichloroacetic acid, metabolites of trichloroethylene, are PPARα activators,[701] and the herbicide Dicamba (2-methoxy-3,6-dichlorobenzoic acid) is another suspected PPAR ligand.[748] Evidence also indicates that the metabolites of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) activate PPARα in mice. These herbicides are peroxisome proliferators *in vivo*, but the parent compounds do not directly activate PPARα or PPAR β/γ,[701] demonstrating a need for metabolic activation.

294. Organotins, such as tributyltin and triphenyltin, are used as antifouling agents, wood preservatives, and in polyvinylchloride plastics. There currently exists a worldwide ban on the use of tributyltin as an antifoulant. These organotins are PPAR β/γ agonists.[7; 403] Tributyltin promotes

adipogenesis in 3T3-L1 cells and increases fat mass in mice exposed during fetal development. Tributyltin has been observed to increase adipocyte number or increase weight in several vertebrate species (see Section 5.3, *Precendent Chemicals*).[7; 403; 698] Interestingly, the organotins also activate PPARs' requisite heterodimeric partner, RXR (see Section 5, *The Retinoid Signaling Pathway*), and it has been hypothesized that the dual action of organotins on both RXR and PPAR β/γ enhances organotin's actions[7] because RXR ligands increase PPAR activity.[677]

8.3.1 PPAR Activators in Non-Mammalian Species

295. Limited information is available on the activation of PPARs in non-mammalian vertebrates by environmentally relevant chemicals. The PPARs of *Xenopus* are activated by many of the same chemicals that activate human PPARs, although PPARβ/δ from *Xenopus* is much more sensitive to bezafibrate than PPARβ/δ from mammals.[637] However, the direct activation of *Xenopus* PPARs by environmental chemicals such as tributyltin, PFOA, and phthalates has not been evaluated. Nevertheless, tributyltin increases ectopic adipocyte formation around the gonads in mice and *Xenopus laevis,* indicating that tributyltin activates *Xenopus* PPARβ/δ *in vivo*.[7] Exposure to butyl benzyl phthalate significantly interferes with normal development in amphibians, and activation of PPARα by butylbenzyl phthalate has been proposed as a probable cause for this toxicity.[749]

296. PPARγ from fish species respond differently to fatty acids as compared to the human ortholog. *Fugu* PPARγ only has two hydrogen bonding residues in its ligand-binding pocket and therefore is unlikely to bind fatty acids with high affinity.[650] Transactivation assays confirm that PPARγ from sea bream or plaice is not activated by fatty acids or typical mammalian PPARγ synthetic ligands.[651]

297. Like PPARs in mammals, evidence exists for a synergistic interaction between ligands in fish to PPAR and its partner RXR. Transactivation assays performed with rainbow trout (*Oncorhynchus mykiss*) PPARs revealed that 50 nM bezafibrate activated PPARβ/δ, but co-treatment with the RXR ligand 9-cis retinoic acid enhanced assay sensitivity to benzafibrate nearly 10-fold.[750]

298. Interestingly, proteomic analysis of arsenic-exposed zebrafish indicated that arsenic activates PPARγ pathways in male zebrafish.[751] Some have speculated that arsenic may be associated with increased risk of diabetes in humans,[752] providing evidence of an important role for comparative studies in human health. Overall, fish PPARs are complex,[753] and evolutionary differences between fish and human indicate that data from mammalian species may not project to keystone environmental species.

8.4 In Vitro Assays

8.4.1 Transactivation Reporter Assays

299. The transactivation assay is the classical reporter assay that demonstrates functional activation of a nuclear receptor by a specific compound[672] (**Table 8-3**). Classical transactivation assays are performed by transfecting a cell of choice with a PPAR expression plasmid and a reporter plasmid. Typically, the reporter plasmid induces the expression of luciferase when the receptor is activated by a chemical and, in turn, binds the reporter's response element.[672; 728; 754-757] Transfection efficiency, a common source of experimental error, can be measured in conjunction with a second reporter (Promega's Dual-Glo assay [Promega, Madison, WI]). Chemical-induced luciferase activity is then normalized to the transfection efficiency found within that specific well. Luciferase activity can then be compared between treated and untreated samples, and antagonism also can be measured. In this case, a precedent ligand is used to activate a PPAR while co-treating with diverse chemicals hypothesized to block activation.

Table 8-3. Screening methods for PPAR activators and inactivators.

Method	*In vivo*/ *In vitro*	Strengths
Transactivation assay	*In vitro*	Demonstrates functional activation or inactivation. Can be easily modified.
3T3-L differentiation	*In vitro*	Demonstrates a physiological response *in vitro*.
Peroxisome Proliferation	*In vivo*	Demonstrates a functional response *in vivo*. Considers metabolism and has been used to demonstrate that a metabolite activates PPAR. Furthermore, the *in vivo* assays demonstrate that a chemical of interest reaches the critical concentrations necessary to activate PPARs.
PPAR-null mice	*In vivo*	Demonstrates a response is mediated through PPARs.
Humanized mice	*In vivo*	Demonstrates a similar function for the human receptor.
Conditional transgenics	*In vivo*	Variety of purposes. Demonstrates specific physiological role of receptors.
Systems Biology (Pathways)	*In vivo*/ *In vitro*	Indicates that specific pathways are activated. May indicate activation of PPARs through novel or unexpected mechanisms.

300. Transactivation assays can be modified by the addition of cofactors and a requisite heterodimeric partner that enhance the sensitivity of the assay, depending on the platform or cells used. For example, SRC-1 or other co-activators can be added to the assay to enhance the sensitivity and demonstrate activation or enhanced activation in the presence of a specific co-activator. These assays are similar to mammalian two-hybrid or yeast two-hybrid assays that demonstrate protein-protein interaction and are called co-activator-dependent receptor ligand assays (CARLA).[758]

301. There are also other methods that have been used to measure PPAR activity, such as the ligand induced complex assay (LIC), which has some similar attributes to the Electrophoretic Mobility Shift Assay (EMSA) and the Differential Protease Sensitivity Assay (DPSA).[637] Interestingly, chemicals shown to activate PPAR acatvation are typically receptor ligands.[637] Thus, the transactivation assay provides the most applicable Level 2 assay aimed at identifying the initiating event leading to adverse outcome (**Table 8-3**).

8.4.2 3T3-L1 Cell Differentiation Assay

302. The 3T3-L1 adipocyte differentiation assay is cell based. Briefly, this preadipose cell line can be induced to differentiate and accumulate triglycerides by specific cues that act as an on-off switch[759] (**Table 8-3**). Unlike the other assays, it does not demonstrate that the changes in differentiation are due to PPARs; however, the assay does demonstrate that there is a physiological change caused by the chemical of interest, and it is much easier to perform than *in vivo* studies. PPARγ ligands are one of the cues that induce adipocyte accumulation and differentiation. Furthermore, the addition of PPARγ-specific siRNAs and the subsequent loss of adipocyte differentiation can provide mechanistic insight.

8.5 In Vivo Assays

8.5.1 Peroxisome Proliferation

303. The key biomarker for PPARα activation is peroxisome proliferation;[668-670] therefore, this liver phenotype can be used to demonstrate PPARα activation *in vivo* (**Table 8-3**). PPARα-null mice do not undergo peroxisome proliferation and, therefore, can be used to further demonstrate that the chemical of

interest is a PPARα ligand. Humanized PPARα mice are also available, and these could be used to reduce the risk of extrapolation from rodents to humans.

8.5.2 Lipid Accumulation

304. The key biomarker or physiological change induced by an obesogen is increased weight gain, especially increased weight gain through lipid accumulation (**Table 8-3**). Considering the incredible increase in obesity over the past 30 years, this is a key biomarker for a number of chemicals, not just PPARγ ligands. Weight gain can be measured with or without use of a high-fat diet and can also be performed using other species such as *Xenopus.*[7] We foresee techniques such as dual-emission X-ray absorptiometry (DXA)[760] being helpful in the diagnosis of chemically induced obesity. Conditional knock outs and gain of function transgenics[761] have been produced in animal models, and some of these may help provide further insight on the physiological effects of metabolic disruptors.

8.5.3 Microarrays

305. Systems biology has significantly altered toxicology over the past 10 years. Analysis of specific molecular pathways using microarrays, proteomics, and even metabolomics following chemical treatment has provided key insight into the mechanism of action of numerous chemicals, including PPAR activators[726; 751; 762-765] (**Table 8-3**).

8.6 Strengths, Challenges, and Limitations

306. Several specific challenges have been addressed throughout this review as they pertain to specific receptors or methods. The primary challenges facing PPAR disruptor are significant species differences in responses. For example, peroxisome proliferation has not been observed in humans because humans express PPARα at much lower levels than rodents.[766] Thus, activation of PPARs in rodents does not necessarily reflect similar physiological perturbations in humans. Nevertheless, peroxisome proliferation in rodent models can serve as a biomarker of PPARα activation in an adverse outcome pathway assessment (**Table 8-4**). Furthermore, fish PPARγ has only two hydrogen-binding residues in its ligand-binding pocket and, therefore, probably has a different ligand-binding profile than mammalian PPARγ receptors. Also, there may be unexpected ligands or physiological perturbations in fish, and extrapolation of data from one species to another may not be possible.

307. Additionally, PPARs are permissive partners with RXR. As such, RXR ligands can activate the PPAR complex. This dual regulation of the receptor complex adds a level of uncertainty when establishing an adverse outcome pathway because chemical activation as identity of the receptor target may remain unknown. Additionally, PPARs are permissive partners with RXR. As such, RXR ligands can activate the PPAR complex. This dual regulation of the receptor complex adds a level of uncertainty when establishing an adverse outcome pathway because identity of the receptor target may remain unknown. While chemical interaction with either RXR or PPAR during Level 2 assays (**Table 8-4**) may result in some of the same adverse outcomes, such ambiguity would not interfere with establishing and characterizing endocrine-disrupting toxicity.

Table 8-4. Integration of a tentative adverse outcome pathway and proposed (January 2012) OECD conceptual framework, with most promising assays to detect and characterize chemical effects on the PPAR signaling pathway*.

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: PPAR α,β/δ,γ activation/inhibition	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	PPAR α,β/δ,γ transactivation reporter assays
Tissue-level responses PPARα: peroxisome proliferation. PPARα,β/δ,γ–specific gene regulation PPARγ: preadipocyte differentiation	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (relevant cell-based assays were included in this level) (Relevant cell-based assays were added to this level)	Peroxisome proliferation assay; Cell-based microarrays; Adipocyte differentiation in cultured pre-adipocyte cells
Organ-level responses PPAR-receptor-specific gene expression	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	Microarray analyses of livers from exposed animals (TG 229, TG 230, TG 206, TG 440, TG 441, fish sexual development test, fish life cycle toxicity test, amphibian metamorphosis assay).
Whole organism responses Obesity	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Weight gain in chronically-exposed animals (TG 415,TG 416, TG443, amphibian development, growth and reproduction assay).

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

SUMMARY, CONCLUSIONS, AND CONSIDERATIONS

9.1 Summary and Conclusions

308. The neuro-endocrine system of vertebrates consists of an array of signaling pathways in which messenger molecules transmit information throughout the body to regulate processes, including those involved in metabolism, reproduction, and growth. Most of these pathways have received little to no attention with regards to their susceptibility to perturbation by environmental chemicals and potential adverse health outcomes associated with such perturbations. In this DRP, we provide a discussion of those pathways for which some published information is available on susceptibility to endocrine disruptors and describe assays that may be used to assess potential disruption.

309. Many of the pathways discussed contribute to common apical events. For example, disregulation of glucocorticoid, growth hormone/IGF-1, retinoic acid, and fatty acid signaling processes all can contribute to symptoms of metabolic syndrome. Metabolic syndrome is associated with a number of symptoms, including cardiovascular disease, type 2 diabetes mellitus, and obesity.[375] Age-adjusted estimates indicate that approximately 34% of the U.S. population over 19 years of age meet the criteria for metabolic syndrome.[767] Metabolic syndrome has been associated with exposure to environmental chemicals, although the mechanistic relationship between exposure and disease outcome remains uncertain.[768] The possibility must be considered that simultaneous disruption of multiple endocrine signaling pathways contributes to this condition.

310. Simultaneous disruption of multiple endocrine signaling pathways may be the consequence of exposure to chemical mixtures. However, single chemicals can perturb multiple pathways. For example, BPA can directly impact thyroid hormone and estrogen signaling and can indirectly affect glucocorticoid, growth hormone/IGF-1 signaling through estrogen cross-talk with these pathways. Taken together, these interactions of BPA with endocrine signaling could be responsible for its reported association with metabolic syndrome.[769]

9.1.1 Cross Talk among Signaling Pathways

311. Cross talk is ubiquitous among endocrine signaling pathways. Thus, disruption of one endocrine signaling pathway can impact signaling of another pathway. In addition to the effect of estrogens on glucocorticoid and growth hormone/IGF-1 signaling, as discussed above with BPA, androgen signaling disruptors can also affect glucocorticoid signaling; thyroid hormone and corticosteroid signaling disruptors can impact the somatrotropic axis; and fatty acid signaling disruptors can impact thyroid hormone signaling. Perhaps most notable is the effect of RXR agonists on signaling of permissive partner receptors. RXR agonists have the potential to disrupt signaling mediated by the PPAR (see Section 8, *The Peroxisome Proliferator-Activated Receptor Signaling Pathway*), farnesoid X receptor (FXR), and the liver X receptor (LXR). Little is known of the susceptibility of the latter two signaling pathways to disruption by environmental chemicals. Several of the pathways discussed in this DRP (glucocorticoid, retinoic acid, thyroid, vitamin D signaling pathways) are subject to cross talk involving the AhR and are, accordingly, susceptible to the disrupting effects of AhR ligands, such as some dioxins and PCBs. Cross talk among signaling pathways adds a new level of complexity when attempting to relate chemical effects in screening assays to apical effects in the whole organism.

9.1.2 Assays

312. Assays used to evaluate endocrine disruption described in this DRP fall within five major categories: Transactivation reporter gene assays (Level 2 assays of OECD Conceptual Framework),

hormone-metabolizing enzyme assays (Level 2 assays), cultured cell responses (these assays typically do not evaluate initiating events in AOPs and therefore, when integrating AOPs and the OECD conceptual frame, were assigned to Level 3 assays), microarrays (Level 3), and *in vivo* adverse apical outcomes (Level 4 and 5). Binding assays involving hormone receptors as the chemical binding site provide limited information. It is the view of the authors of this DRP that the cost and time investment into such assays is not worth the benefit, considering that other, more definitive receptor screening assays are available. Protein-binding assays have value in some specific applications, such as evaluating the interactions between a chemical and plasma hormone-binding proteins (see Section 3, *Hypothalamus:Pituitary:Gonad Axis*). Transactivation reporter gene assays, on the other hand, provide quantitative information on the interaction of a chemical with a hormone receptor from a functional standpoint (agonist or antagonist activity). Furthermore, reporter assays are commercially available for many of the nuclear receptors (**Table 9-1**) and these assays have known performance capabilities (e.g., sensitivity, coefficient of variation). The use of reporter assays to screen chemicals for interaction with nuclear receptors is recommended. Chemical-induced perturbations in endogenous hormone levels are another common initiating event leading to adverse outcome. Examples include the depletion of glucocorticoid, thyroid hormone, and retinoid stores (see Sections 2, 6, and 7). Precedents exist for assaying such perturbations in cultured cells (i.e., modifications to U.S. EPA OCSPP 890.1550). Alternatively, analyses of hormone levels can be added to existing Test Guidelines involving whole animal exposures.

313. Screening assays involving cultured cells can account for additional complexities within relevant cell-types by assaying the normal function of the cells as related to the endocrine signaling pathway under investigation. Some assays described require the isolation of primary cells from animals, but many cell-based assays described herein utilize established cell lines (**Table 9-2**). Like reporter assays, these cell-based assays are relatively simple to perform, are time and cost effective, and cell propagation methods have been standardized.

314. Microarrays involve the analysis of changes in gene expression (mRNA levels) for massive numbers of genes following exposure of cells or whole organisms to the chemical of interest. Strengths of the approach include the simultaneous analyses of components along the signaling pathway, as well as products of the signaling pathway. The approach also allows for the simultaneous analyses of multiple signaling pathways. However, the assays require the construction of the arrays, which can be cost and time intensive (some are commercially available, but are relatively expensive); require challenging analyses of the mass of data generated and often suffer from lack of reproducibility. The potential ability to assess chemical impact on multiple endocrine signaling pathways simultaneously is one of the greatest strengths of microarrays. However, the approach may not be sufficiently developed for routine, validated use at this time.

315. Many apical endpoints that have been described in this DRP could be added to currently recommended whole-organism assays for the assessment of disruption of additional endocrine pathways. Such endpoints are summarized in **Table 9-3**. Several of these approaches involve the analysis of serum/plasma hormone levels or products of the pathway. The development of analytical approaches that could be used in the mass analyses of these molecules would provide significant additional information to some standard whole organism assays. Since IGF-1 levels are influenced by the estrogen, androgen, thyroid, and corticosteroid signaling pathways, analyses of IGF-1 levels in whole organism assays could provide insight into endocrine disruption involving one or several pathways. Consequences of IGF-1 disruption would be impaired growth, which is a common outcome of exposure to environmental chemicals.

Table 9-1. Some commercially available reporter gene assay kits for use to screen chemicals for interactions with nuclear receptors.

Nuclear Receptor	Commercial Source
Glucocorticoid (GR)	▪ Panomics/Affymetrix ▪ Indigo Biosciences ▪ Qiagen/SABiosciences
Androgen (AR)	▪ Qiagen/SABiosciences
Estrogen (ER)	▪ Qiagen/SABiosciences ▪ Indigo Biosciences ▪ BioDetection Systems
Progesterone (PR)	▪ Qiagen/SABiosciences
Retinoic acid (RAR)	▪ Qiagen/SABiosciences
Retinoid X (RXR)	▪ Qiagen/SABiosciences
Thyroid (TR)	▪ Indigo Biosciences
Vitamin D (VDR)	▪ Qiagen/SABiosciences
Peroxisome Proliferator- Activated (PPAR)	▪ Qiagen/SABiosciences
Aryl Hydrocarbon (AhR)	▪ Qiagen/SABiosciences ▪ Indigo Biosciences ▪ BioDetection Systems

The list of vendors was generated by the authors and does not represent an endorsement by the OECD.

Table 9-2. Cell-based assays used to assess disruption of endocrine signaling processes by exogenous chemicals.

Signaling Pathway	Cells	Endpoint
Glucocorticoid (GR)	▪ Corticotropes (primary) ▪ Adrenal cortical (primary)	▪ ACTH release ▪ Adrenal hormone release
Somatotropic	▪ Trout testicular (primary)	▪ ^{3}H-thymidine incorporation ▪ IGF-1 binding
Thyroid hormone	▪ Purkinje (primary) ▪ Granule cells (primary) ▪ GH3 (established) ▪ FRTL-5 (established)	▪ Dendritic arborization ▪ Neurite extension ▪ Proliferation ▪ Iodine uptake
RXR/PPAR	▪ 3T3-L1 (established) ▪ C3H10T1/2 (established)	▪ Differentiation ▪ Differentiation

Table 9-3. Some apical endpoints that could be applied to currently recommended whole organism assays to assess disruption of additional endocrine signaling pathways.

Signaling Pathway	Endpoint
Adrenal	▪ Stress responses
Androgen/estrogen	▪ Reproductive behavior ▪ GnRH and aromatase expression
Somatrophic	▪ Plasma IGF-1 levels ▪ Weight and length of organism
Retinoic acid	▪ Retinoid levels ▪ Lipid levels
Thyroid hormone	▪ Thyroid hormone levels ▪ Thyroid gland histology ▪ Thyroid hormone-responsive gene expression
Vitamin D	▪ Plasma vitamin D and metabolites levels ▪ Bone morphology
Fatty acid (PPAR)	▪ Hepatic peroxisome proliferation ▪ Lipid accumulation

316. Among the "-omic" approaches for assessing responses to EDCs, microarrays have been most extensively used. Microarrays have proven utility in evaluating pathway responses (gene induction/suppression) following exposure of cultured cells or whole organisms to a chemical. Often, such pathway changes have correlated well with phenotypic responses. Such use is noteworthy as related to effects of BPA and PCBs on frog metamorphosis (see Section 6, *The Hypothalamus:Pituitary:Thyroid Axis*). Further, the use of microarrays holds the potential to evaluate multiple endocrine pathways for disruption simultaneously by selecting appropriate cell or tissue types for analyses. Such simultaneous analyses would provide a wealth of information on individual pathways, but would also provide insight into disruption involving cross-talk among pathways. However, some microarray studies have revealed poor reproducibility of pathway responses; for example, as related to retinoid/RXR signaling (see Section 5, *The Retinoid Signaling Pathway*). Microarray may prove to be powerful indicators of responses to EDC exposure, but their specific application will require significant standardization and validation.

9.2 Next Steps for Further Consideration

317. All of the endocrine-signaling pathways described in this DRP warrant consideration for inclusion in a program designed to identify EDCs. However, the level of assay development varies among the pathways and some are thus better positioned for incorporation in the existing testing paradigm while others require more research and assay development before they should be considered.

9.2.1 Hypothalamus:Pituitary:Adrenocortical Axis

318. The HPA axis contributes to many physiological processes, including maintenance of lipid and glucose homeostasis, brain function, osmotic balance, and integrity of the immune response and stress response. Symptoms of dysfunction include obesity, metabolic syndrome, diabetes mellitus, immunodeficiency, and improper stress response. However, little precedence exists for the use of endpoints related to these processes in assessing *in vivo* HPA disruption. Several *in vitro* assays exist that could be used to evaluate disruption of corticosteroid signaling including reporter assays and cultured cell

responses. However, little precedent exists for the use of these assays in evaluating chemical effects on this pathway. Assay refinement and validation are required for both *in vitro* and *in vivo* assays/endpoints prior to their incorporation into new or existing Test Guidelines (**Table 9-4)**. Furthermore, the important role of this axis in the stress response adds complexity in establishing whether excitation of the signaling pathway during *in vivo* assessments represents a consequence of endocrine disruption or a normal response to the stress associated with chemical exposure. Thus, establishing linkages between *in vivo* effects and this pathway would rely heavily upon *in vitro* mechanistic assays to establish plausibility of *in vivo* endocrine toxicity.

Table 9-4. Assays to be considered for for the detection of EDCs.

Signaling Pathway	Assays
HPA	▪ glucocorticoid reporter asay (*in vitro*) ▪ ACTH release (*in vivo*) ▪ Adrenal steroid synthesis (*in vitro*, *in vivo*) ▪ Stress response (*in vivo*)
HPG	▪ Assay development required
Somatotropic	▪ IGF-1 levels (*in vivo*) ▪ Growth (*in vivo*)
Retinoid	▪ RXR reporter assay (*in vitro*) ▪ RAR reporter assay (*in vitro*) ▪ AhR reporter assay (*in vitro*) ▪ Adipocyte differentiation (*in vitro*) ▪ Lipid accumulation (*in vivo*) ▪ Serum retinoid levels (*in vivo*)
HPT	▪ TR reporter (*in vitro*) ▪ Cell proliferation (*in vitro*) ▪ Thyroid peroxidase (*in vitro*) ▪ Iodide uptake (*in vitro*)
Vitamin D	▪ Assay development required
PPAR	▪ Transactivation reporter (*in vitro*) ▪ Adipocyte differentiation (*in vitro*) ▪ Peroxisome proliferation (*in vivo*) ▪ Lipid accumulation (*in vivo*)

9.2.2 Hypothalamus:Pituitary:Gonadal Axis

319. Estrogen and androgen signaling pathways are currently major components of OECD's EDC Test Guidelines. However, current Test Guidelines do not include some considerations, such as signaling via membrane receptors, nor do Test Guidelines address disruption of gestagen signaling. Adverse outcome pathways relating to these components of the HPG signaling pathways are poorly developed and, accordingly, relevant endpoints for use in assays are poorly understood. Additional research is needed to advance our understanding of the structure of these adverse outcome pathways, the susceptibility of the pathways to endocrine disruption, and the *in vivo* significance of such disruption.

9.2.3 Somatotropic Axis

320. The somatotropic axis holds promise in assessing endocrine disruption associated with chemical exposure because several endocrine signaling pathways converge on this pathway. Accordingly,

disruption of androgen, estrogen, corticosteroid, and thyroid signaling could be detected by measuring alterations in circulating IGF-1 levels during *in vivo* assays (**Table 9-4)**. Thus, while not diagnostic of a specific mode of action, changes in IGF-1 levels could be added to whole organism screening assays to determine the occurrence of endocrine disruption in general or could be applied to longer-term, whole animal exposures to detect overt endocrine disruption during these exposures. This endpoint may be of use to confirm or refute endocrine disruption that is suggested by mechanistic *in vitro* assays. This endpoint would require assay development and validation prior to its adoption since IGF-1 levels can be influenced by a variety of exogenous factors (e.g., food, temperature, photoperiod) and the endpoint has not be extensively used to assess endocrine disruption. Alterations in the somatotropic axis also can be detected by monitoring weight and length of fetal rodents and growth rates in fishes. These endpoints could be added to several current OECD Test Guidelines.

9.2.4 Retinoid Signaling Pathway

321. RXR functions as a central node in regulating various facets of reproduction, development, and lipid homeostasis through its heterodimerization with other nuclear receptors. Among its heterodimer partners are PPAR, TR, VDR, and the RAR. The RXR has been shown to be highly susceptible to activation by some xenobiotics, such as tributyltin, resulting in alterations in lipid homeostasis and intersex conditions in some invertebrates. RXR is expressed in almost all faunal species thus far examined. Transactivation reporter assays are commercially available for RXR and RAR. In addition, AhR agonists have the ability to deplete retinoid levels, thus disrupting this signaling pathway. AhR reporter assays also are commercially available and should be included in the conceptual framework (**Table 9-4)**. Adipocyte differentiation assays, as described for PPAR, also are information with regards to RXR since RXR agonists can activate the RXR:PPAR complex, resulting in alterations in adipocyte differentiation and lipid accumulation. Serum retinoid levels can be informative in whole-animal exposures since AhR ligands can deplete retinoid levels and disrupt normal retinoid signaling.

9.2.5 Hypothalamus:Pituitary:Thyroid Axis

322. OECD Test Guidelines currently exist for several *in vitro* and *in vivo* assays that are relevant for the detection and assessment of disruption of the HPT axis. Additional assays could be considered that would strengthen the linkage between initiating events and adverse apical effects along the adverse outcome pathway (**Table 9-4)**. Transactivation reporter assays and cell proliferation assays are available that would definitively evaluate the ability of xenobiotics to bind the thyroid hormone receptor and function as an agonist or antagonist. The thyroid peroxidase inhibition assay and the iodide uptake assay both could provide information on thyroid hormone signaling disruption in a screening format. Assessment of HPT-regulated gene expression could be incorporated into existing *in vivo* assays designed as a linkage between molecular events and apical outcomes under the same experimental design.

9.2.6 Vitamin D Signaling Pathway

323. Vitamin D plays important roles in the development and maintenance of various systems, including bone, immune, cardiac, and neurological. Despite its important role in overall well-being, few studies have been performed that directly assess the impact of chemical exposure on this signaling pathway. Studies typically have evaluated chemical effects on some apical endpoint (i.e., bone development), which may or may not be related to effects on vitamin D signaling. Studies, to date, indicate that the VDR is highly specialized with respect to ligand binding, and xenobiotics typically do not bind to the receptor. More likely, disruption would be caused by effects of chemicals on the metabolic enzymes responsible for vitamin D synthesis. Again, little data are available to support this premise. Additional studies are warranted to evaluate the susceptibility of vitamin D anabolic and catabolic

enzymes (CYP2R1, CYP27B1, CYP24A1) to interaction with exogenous chemicals, as this may prove to be a viable cause of endocrine disruption.

9.2.7 Peroxisome Proliferator-Activated Receptor Signaling Pathway

324. The PPAR pathway, which is typically activated by fatty acids, is clearly involved in lipid and glucose homeostasis, inflammation, and aspects of development. The adverse outcome pathway involving PPARγ is reasonably well established, with activation of the receptor leading to adipocyte differentiation, lipid accumulation, and weight gain. The adverse outcome pathway involving PPARα is less well defined, and little is known of the adverse outcome pathway involving PPARβ/δ. Perturbations in this pathway by environmental chemicals can have detrimental effects consistent with metabolic syndrome and other health conditions facing modern society. Assays that could be used to assess disruption of normal signaling have been well developed. Screening assays are available for the rapid assessment of PPAR signal disruptors, as are apical endpoints that could be incorporated into currently recommended OECD whole animal assays (**Table 9-4)**. Among screening assays, prioritization should be given to PPAR transactivation reporter assays and adipocyte differentiation assays. Prioritization also should be given to incorporating peroxisome proliferation and lipid accumulation into OECD-recommended whole organism assays.

9.2.8 Adverse Outcome Pathway approach and the Conceptual Framework

325. Further discussion on the AOPs and allocation of the methods to the various levels of the CF and the connection to the AOP is recommended in order to further streamline and focus the discussion on the various pathways.

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ANNEX 1: ENDOCRINE DISRUPTERS AND THE EPIGENOME

1.0 Introduction

1. The mechanism by which the group of chemicals referred to as 'endocrine disruptors' exert their phenotypic effects remains only partially understood, but there is emerging evidence that dysregulation of the cell's epigenome is involved. In the last decade, it has become clear that the emerging field of epigenetics is of significant relevance for both the study and practice of toxicology and safety assessment. At the research level, these efforts currently aim to elucidate the involvement of chemical-induced epigenetic changes in adverse health effects, as well as to the exploitation of epigenetics particularly in the area of *in vitro* and *in vivo* modeling. While there have been plenty of reports linking endocrine disruptors with phenotypic abnormalities in wildlife, there are currently no publications describing epigenetic studies in wildlife undergoing these exposures. While wildlife and ecotoxicological aspects are not specifically addressed in this chapter, some of the models discussed may be relevant. This chapter reviews our current understanding of the intersection of these two fields of research and proposes avenues of exploration encompassing epigenetic information that will form the foundation for definitive testing of this relationship and provide a basis for future practical applications for regulatory safety assessment.

2. The issue of incorporation of epigenetic evaluations into safety assessments has been reviewed in recent times (Goodman, Augustine et al. 2010), with the conclusion that the rapidly-developing field of epigenetics shows promise as a means of gaining insights into the effects of endocrine disruptors upon human health, but that there remains a lot to be learned before it is clear how these tests should be applied. How eventually to incorporate the understanding of epigenetic mechanisms into the OECD chemical safety assessment regulatory activities and how this might be done within the ED conceptual framework screening, priority and definitive testing levels, is the major challenge and objective that this chapter will begin to explore and address..

2.0 Definitions

2.1 The epigenome

3. The word epigenome is derived from epigenetics, a term attributed to Waddington (Waddington 1942) who defined it as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being". Waddington was looking for an explanation of how the same genome could be used to generate different cell types in multicellular organisms, suggesting a higher level of regulation acting on non-autonomous genes. The term epigenetic was resurrected more recently as a broad description of heritable processes that do not depend on changes in DNA sequence, to include phenomena such as genomic imprinting and X chromosome inactivation. In each of these examples, a locus on one of the two homologous chromosomes almost identical (or completely identical in inbred mouse strains) in terms of DNA sequence is silenced, with the other active, a state that remains stable from parent to daughter cells, thus the heritability component.

4. Some of the molecular mechanisms implicated in allelic silencing includes methylation of DNA (Sapienza, Paquette et al. 1989), histone modifications and variant deposition (Delaval and Feil 2004), DNA replication timing (LaSalle and Lalande 1996), antisense and non-coding RNA transcription (Whitehead, Pandey et al. 2009) among others (see below). Of these, only DNA methylation had a demonstrable biochemical mechanism for parent to daughter cell propagation of its regulatory message

(maintenance DNA methyltransferase, DNMT1 (Goyal, Reinhardt et al. 2006), making DNA methylation the standard bearer for an epigenetic regulator, but this is mostly because of a current dearth of knowledge about how other mechanisms may be heritable, which may in time be revealed but at present prove elusive.

5. "Epigenome" represents the collective noun to describe the sum of the epigenetic modifications throughout the genome. This is where the common use of the term deviates from the strict definition, as the term describes molecular mediators and not heritable influences on cellular properties. As such, the term describes a broad group of transcriptional regulatory processes, of which only DNA methylation is demonstrably heritable. This incorrect use of the term is, however, useful, as there is no other obvious term that describes the broad group of transcriptional regulatory processes including chromatin and DNA properties that gets across the idea that some of these properties may mediate a cellular memory.

6. Acknowledging that the use of the term epigenome or epigenomic is inherently flawed in terms of its origins, this chapter will utilise the term as commonly and incorrectly used to describe the full spectrum of transcriptional regulatory processes that appear to mediate environmental influences and change a cellular state to reflect past exposures.

2.2 Epigenomic regulatory mechanisms

7. The molecular mechanisms believed to mediate epigenetic and transcriptional regulation are diverse (summarized in **Table 1**).

Table 1: Examples of molecular regulators of the vertebrate epigenome.

Molecular mediator	Example	References
Histone post-translational modifications	Histone H3 lysine 9 trimethylation (H3K9me3), a repressive mark	(Hiragami-Hamada, Xie et al. 2009)
Histone variants	Histone Macro H2A.1	(Bernstein, Muratore-Schroeder et al. 2008)
Nucleosome positioning	Nucleosome-free regions at gene promoters	(Hartley and Madhani 2009)
Chromatin looping	*Kit* regulation by Gata1/Gata2	(Jing, Vakoc et al. 2008)
DNA modifications	Cytosine methylation	(Klose and Bird 2006)
DNA structural variation	R-loop formation	(Roy, Yu et al. 2008)
RNA-mediated	Antisense RNA transcription	(Beiter, Reich et al. 2009)

8. What these regulators have in common is a lack of innate DNA sequence specificity (with the possible exception of certain DNA methyltransferases which may preferentially target certain CG dinucleotide periodicities (Jia, Jurkowska et al. 2007)). To exert sequence-specific events, it is likely that transcription factors and other DNA-binding proteins with sequence preferences help to recruit modifying enzyme complexes (Beckerman and Prives 2010), one of the ways that the boundary between

transcriptional and epigenetic regulators blurs in terms of functions. Another source of sequence-specificity may be the endogeneous short interfering RNAs (siRNAs) that have been found to induce heterochromatinisation in plants and yeast (Pikaard 2006; Zofall and Grewal 2006). Although there has been little evidence for such mechanisms in mammalian cells in the past (Morris, Chan et al. 2004; Kim, Villeneuve et al. 2006), there is now increasing evidence for siRNA induced heterochromatin in mammals too (Ahlenstiel et al., 2011; Santenard et al., 2010).

9. As mentioned earlier, only DNA methylation has a molecular mechanism defined that allows it to act in a heritable manner from parent to daughter cells. The DNA methyltransferase 1 enzyme (DNMT1) has the ability to recognize (with the chromatin protein UHRF1) loci where a symmetrically-methylated CG dinucleotide (methylation on both the Watson and Crick strands) becomes hemi-methylated following DNA replication (which introduces an unmethylated cytosine when creating the new complementary strands of DNA) and restores the locus to symmetrical methylation on both daughter chromatids. This maintenance methyltransferase function thus allows a methylation mark in a parent cell to be maintained in both daughter cells.

10. The stability of other putative epigenetic regulators in populations of growing cells suggests that they can also maintain themselves in a site-specific manner through DNA replication, potentially through the association of enzymes with chromatin through DNA replication, as demonstrated using an *in vitro* system (Francis, Follmer et al. 2009). RNA-mediated effects such as paramutation, best described in plants (Chandler 2007), have been observed in mice (Rassoulzadegan, Grandjean et al. 2006), although it is unclear how RNA molecules can self-replicate in mammals which appear to lack the RNA-dependent RNA polymerase needed for paramutation in plants (Alleman, Sidorenko et al. 2006).

11. While the molecular basis for the maintenance of epigenomic marks at a locus in dividing or post-mitotic cells remains largely unknown, the stability of these marks is well-recognised and suggests that the failure to find maintenance mechanisms does not mean that they do not exist but that they are eluding our scrutiny.

2.3 Influences exerted by epigenomic regulatory mechanisms

12. The primary means by which the genome communicates its information is through transcription, so it should not be surprising that the major outcome of epigenomic regulators is usually viewed as gene expression. When histone post-translational modifications are referred to as active or repressive marks, it is in terms of gene expression locally.

13. It becomes more complicated – the relationship of a chromatin mark with a gene activity is also dependent on the genomic context of that mark. For example, the histone H3 lysine 9 trimethylation (H3K9me3) mark is recognized as repressive, associated with heterochromatin on a cytological scale (Peters, Mermoud et al. 2002) and with gene silencing when present in the context of a gene promoter. However, the same modification is found to be enriched in the bodies of actively-transcribed genes (Vakoc, Mandat et al. 2005), the opposite correlation in a different genomic context. The same has been found for DNA methylation, increased at promoters of silent genes but also increased in the transcribed bodies of highly-expressed genes (Ball, Li et al. 2009). This contextual information is important when defining relationships of epigenomic regulatory marks and transcription.

14. Epigenomic regulation has also been associated with other genomic properties. DNA replication occurs at different times in the cell cycle in different genomic regions, with specific patterns of timing defining some regions as early and others late-replicating. Even at the cytological scale it is apparent that silencing marks are enriched at later-replicating regions and *vice versa*. Meiotic recombination in humans has been linked to germline DNA methylation patterns (Sigurdsson, Smith et al. 2009), and has been more precisely mapped to areas of open chromatin in yeast (Kauppi, Jeffreys et al. 2004). Decreased global DNA methylation in mammalian cells has been linked causally to chromosomal instability (Karpf

and Matsui 2005), while mutations of the *DNMT3B* maintenance DNA methyltransferase causes distinctive chromosomal morphological abnormalities (Hansen, Wijmenga et al. 1999). The highly abnormal nuclear morphology of B lymphocytes infected with Epstein-Barr virus reflects a profound disturbance of DNA methylation globally in these cells (Grafodatskaya, Choufani et al. 2010), indicating that even cytological-scale morphology has regulatory input by these epigenomic mediators.

15. Recently there has been an unexpected relationship revealed between chromatin organization (Vakoc, Mandat et al. 2005) or DNA methylation (Laurent, Wong et al. 2010) and the exonic organization of genes. This is unexpected because at the stage of generation of the primary transcript the gene might be expected to be agnostic regarding where splicing is occurring, an event that occurs distantly from the gene within the nucleus, an assumption being refined in recent years (Schwartz and Ast 2010). In spite of this, the patterns of nucleosomal positioning (Tilgner, Nikolaou et al. 2009) and DNA methylation observed at intron/exon boundaries have been shown to be distinctive (Laurent, Wong et al. 2010). This raises the possibility that epigenomic regulators could be influencing splice isoform choices made in a cell type, which could have significant functional consequences for the cell. This relationship has yet to be proven rigourously, but represents an intriguing avenue of exploration.

2.4 Large-scale studies of the epigenome

16. With the large number of regulators involved, each causing potentially different organization not only in the several hundred cell types within the body but also in the same cell types over time and in different sexes (Fraga, Ballestar et al. 2005; Thompson, Atzmon et al. 2010), it is clear that there is a very large number of potential epigenomes for each organism. As a further complicating factor, we do not understand how to interpret many of the regulatory marks in different genomic contexts, so that even if we could catalogue epigenomes, understanding their meaning would remain difficult.

17. With these issues in mind, there are several large-scale initiatives to study epigenomic organization. The ENCyclopedia Of DNA Elements (ENCODE) is a project focused on understanding the function of non-coding DNA sequences in the genome, starting originally with transformed human cell lines and expanding through the modENCODE project to include primary cells from model organisms. This project has involved technology development, a lot of mapping, and insights through the development of new, sophisticated analytical approaches. This created a foundation for the Roadmap in Epigenomics, which was set up to differ in terms of a focus on primary, non-diseased human cell types, but also includes technology development and analytical aspects. The Cancer Genome Atlas (TCGA) represents another substantial project that includes an epigenomic component, but the focus in this case is not solely the epigenome. Finally the International Human Epigenome Consortium (IHEC) is in an early stage of development but plans to bring the Roadmap in Epigenomics concept a step further by looking specifically at human diseases. **Table 2** lists these initiatives and web-based resources for the reader to explore further.

18. These projects are now productive and provide insights into how epigenomes are organized, and how epigenomic information interacts with genetic polymorphism (Kasowski, Grubert et al. 2010; McDaniell, Lee et al. 2010). While insights into the epigenomic organisation of a specific human cell type can be gained from the Roadmap project, it should be stressed that these studies tend to be deep (many assays performed on a single cell sample) rather than broad (testing many cell samples), and there are relatively few metadata captured about the donors, making these data unsuitable for most human disease or exposure studies.

Table 2: Large-scale studies studying epigenomic organization.

Project	Abbreviation	Web resources
ENCyclopedia Of DNA Elements	ENCODE, modENCODE	http://www.genome.gov/10005107 http://genome.ucsc.edu/ENCODE/ http://www.modencode.org/ http://www.genome.gov/modencode/
Roadmap in Epigenomics		http://www.roadmapepigenomics.org/ http://www.epigenomebrowser.org/ http://www.ncbi.nlm.nih.gov/epigenomics
The Cancer Genome Atlas	TCGA	http://www.genome.gov/17516564 http://cancergenome.nih.gov/ http://tcga-data.nci.nih.gov/tcga
International Human Epigenome Consortium	IHEC	http://www.ihec-epigenomes.org/

2.5 Genome-wide assays: the transition from microarrays to massively-parallel sequencing

19. Genome-wide study, in essence, involves enriching a fraction of the nucleic acid in the cell and determining where in the genome the nucleic acid came from. Gene expression microarrays represent a well-known paradigm for genome-wide assays. To perform these studies, RNA from the cell is isolated and hybridized to short DNA sequences immobilized on a glass slide. These short DNA sequences are designed to represent each gene in the genome. The RNA is converted to DNA and labeled with fluorescent molecules, so that the presence of a specific gene's RNA in the pool isolated from the cell sample will cause the short DNA sequence on the glass slide to acquire a fluorescent signal proportional to the amount of RNA labeled. As the location of the DNA sequences on the slide are pre-defined, scanning the slide to look at relative fluorescence of each DNA sequence location can be converted to a gene expression measure for each gene represented. Chromatin immunoprecipitation (ChIP) can likewise be performed with microarrays, but the starting material differs (immunoprecipitated DNA) and the DNA sequences on the slide also differ (representing regions of interest like gene promoters, for example). Massively-parallel sequencing differs by taking the RNA or immunoprecipitated DNA and performing sequencing of the molecules, so that relative enrichment of a certain gene's expression or a chromatin component is measured not by fluorescence intensity but by the relative amount of sequence mapped to a specific gene or location.

20. Microarray technology matured years before massively-parallel sequencing (MPS) and remains a significant means of investigation of the epigenome and transcriptome. Microarrays have some problems, both technical and financial. From a technical perspective, it was noted that the signal/noise discrimination and dynamic range of signal associated with MPS-based detection greatly exceeds that of identical assays performed using microarrays (Suzuki, Jing et al. 2010). From a cost perspective, the price per unit of DNA sequence length is now much less for MPS, but an advantage still retained by microarrays is the ability to study only a limited subset of the genome, which still makes such studies more affordable for microarrays, although the rationale for such limited studies is decreasingly warranted. Furthermore, the data characteristics from MPS-based assays are substantially different from those generated by microarrays – the sequence information allows allelic discrimination, alternative splicing

detection, nucleotide resolution DNA methylation studies, and information from as yet unsequenced regions of the genome, making MPS data potentially of even greater value with time.

21. All massively-parallel sequencing technologies to date involve the sequential addition of nucleotides to immobilised target DNA sequences, detecting the events usually through distinctive fluorescence signals and light microscopy but more recently also through hydrogen ion release (http://www.iontorrent.com/). The technologies thus far involve a tradeoff between shorter (≤200 bp) sequences but more of them (hundreds of millions) per machine run, or the opposite, longer (≥500 bp) but fewer (≤1 million) sequences per run. The trend of sequencing technologies is towards continued rapidly growing capacity, with decreasing costs per unit length of DNA sequence, with the oft-stated benchmark goal of a $1,000 genome (Mardis 2006). The use of MPS is likely to continue to expand beyond even that of today, leading to profound new insights but also the data challenges summarized later in **section 6**.

2.6 The problem of choice in epigenomic assays

22. When cells with an identical genome are compared for epigenomic differences following exposure to different toxins, it is apparent even from **Table 1** that there are many potential mediators of epigenomic organization, and frequently no indication which one that can be assumed to be informative *a priori*. If anything, **Table 1** vastly oversimplifies the problem – histone H3 lysine 9 trimethylation (H3K9me3) is only one of hundreds of post-translational modifications of the canonical core histones (H2A, H2B, H3 and H4) (Bannister and Kouzarides 2011), before the many histone variants are considered, the positioning of the nucleosomes they assemble, the influence of DNA methylation on the DNA they package, and so on. While our focus in this review is on DNA methylation, it should also be acknowledged that 5-methylcytosine is only one of several cytosine variants now recognized to include 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (He, Li et al. 2011). It is therefore extremely difficult to choose the most appropriate assay for a given question, and the cost and cell quantities required for these studies remain sufficiently substantial that a scattershot approach is not an option.

23. In practice, the choice is often constrained to a focus on DNA methylation studies for a number of reasons. There is generally more familiarity with the assays involved. The sample requirements are usually less onerous (in terms of quantity and preparation) than for RNA or chromatin-focused studies. Furthermore, the assays are demonstrably quantitative, something that has yet to be shown for ChIP followed by MPS (ChIP-seq), an important issue discussed later in **section 5**.

24. A significant problem with DNA methylation is that we don't really know how to interpret many of the observed non-promoter changes, and the correlation of DNA methylation with local gene expression changes is far from straightforward. This kind of consideration has kept ChIP-seq of major interest to researchers of human disease, prompting attempts to miniaturise the assay in terms of sample requirements (Adli, Zhu et al. 2010). Furthermore, as many chromatin components with regulatory associations appear to have redundancy in terms of genomic location and transcriptional function, it appears that it may not be necessary to survey all possible chromatin marks. This hypothesis was tested as part of the ENCODE project (Ernst and Kellis 2010). They found that certain combinations of chromatin marks or constituents were able to predict regulatory function, and that much of this information could be captured by a subset of the 41 that they tested. This indicates a means by which we may be able to make some informed choices about how to study this large number of regulators when performing epigenomic studies.

3.0 Potential effects

25. The reason for interest in the role of the epigenome is based on several observations. Firstly, as will be described next (**section 4**), there is emerging evidence that sex steroid receptor activity exerts consequential effects by means of some of the epigenomic and transcriptional regulatory processes outlined earlier. Secondly, the field of endocrine disruptor effects is notable for transgenerational consequences – a risk of disease in the unexposed progeny of exposed parents. When this kind of cellular memory event occurs, the obvious question is how such a memory is mediated at the molecular level. The epigenome has properties as described earlier (**section 2**) that allow it to be considered as a candidate for mediating such long-term memory mechanisms. Thirdly, there are now several studies that link known endocrine disruptors to effects on the epigenome (**section 4**) that offer more direct evidence for mechanistic associations.

26. A model for conceptualising the mechanism by which endocrine disruptors exert their effects might be proposed as follows. Sex steroid hormones have their effects mediated in part through epigenomic and transcriptional regulators. This induces long-lasting changes in cellular states that we recognize to be due to normal sex hormone exposure. The long-term maintenance of these new cellular states relies in part upon epigenomic reorganization. The exposure to endocrine disruptors causes similar or distinct effects on cellular states, again mediated in part by epigenomic reorganization. This epigenomic reorganization is not the same as that mediated by endogeneous sex steroids, in terms of timing and perhaps the type of epigenomic changes themselves. There is furthermore a possibility that the epigenomic changes induced by endocrine disruptors are unusually stable, long-lived, and widespread enough in terms of target cell types that gametes become involved and mediate transgenerational inheritance of these changes, with phenotypic consequences.

27. This hypothesis is based upon the observations of the next two sections, but it should be stressed that direct evidence, especially in humans, remains only partial. As a consequence, while we have sufficient evidence to be concerned about the epigenome mediating pathogenic effects of endocrine disruptors, we lack definitive proof that this is the sole or even major means by which these environmental agents cause human disease consequences.

28. From a different perspective, in the field of lung cancer, the use of DNA methylation-based biomarkers detectable in for example peripheral blood has emerged as a highly promising method complementing imaging techniques, and these biomarkers are now being actively studied in multiple cancers (Anglim et al., 2008).

4.0 Evidence for endocrine disruption being mediated by epigenomic processes

29. The relationship between the epigenome and epigenetic regulation has mostly been studied in terms of how genes involved in endocrine signaling are themselves regulated by epigenetic processes such as DNA methylation. This has been reviewed comprehensively (Zhang and Ho 2011) and allows the data of **Table 3** to be presented as a summary of the state of this field.

30. A more relevant process from the perspective of this review is the effect that endogenous hormones have on the epigenome, so that we can understand how endocrine disruptors may exert their effects. This has also been studied reasonably comprehensively and has been reviewed in detail recently (LeBaron, Rasoulpour et al. 2010; Vandegehuchte and Janssen 2011; Zhang and Ho 2011). A major reason for considering a link between endocrine disruptors and the epigenome is because the action of certain hormones is mediated in part through epigenetic regulators. As described in previous chapters of this DRP, the molecular mechanisms of endocrine active substances may involve different pathways including interactions with nuclear hormone receptors. Retinoic acid, steroid hormones, calcitriol and thyroid hormone bind to nuclear receptors within the cell and enter the nucleus to bind as a complex to hormone-

responsive elements (Evans 1988). For example, when the thyroid hormone receptor binds to a response element, the nuclear receptor can do so on its own, in which case it appears to act as a transcriptional repressor, recruiting a multi-protein complex that includes histone deacetylase and SIN3A, whereas if the thyroid hormone receptor binds as a heterodimer with another nuclear receptors such as retinoid X receptors it activates transcription by recruiting the histone acetyltransferases PCAF and CBP (Zhang and Lazar 2000). Recently, several hundreds of potential transcriptional coregulators that interact directly and indirectly with nuclear receptors have been identified (O'Malley, Qin et al. 2008; Kato et al., 2011), including fat-soluble ligands like vitamin A/D and steroid hormones receptors and peroxisome proliferator-activated receptor gamma (PPARγ), which plays critical roles in metabolism and adipogenesis (Kato and Fujiki, 2011b; Sugii and Evans, 2011). Genes that represent specific targets of oestrogen receptor alpha have been identified using the chromatin immunoprecipitation approach (Jin, Leu et al. 2004; Lin, Reierstad et al. 2007), allowing insight into the downstream effectors of hormonal signaling.

Table 3: Mammalian endocrine genes regulated by DNA methylation (from (Zhang and Ho 2011))**.**

Gene name	**Gene symbol**
P450scc	*CYP11A1*
3ß-hydroxysteroid dehydrogenase	*HSD3B1/2*
17α-hydroxylase	*CYP17A1*
17ß-hydroxylase	*HSD17B3*
Vitamin D synthesis	*CYP27A1/B1*
Androgen receptor	*AR*
Oestrogen receptor 1	*ESR1*
Oestrogen receptor 2	*ESR2*
Progesterone receptor	*PGR*
Glucocorticoid receptor	*NR3C1*
Mineralocorticoid receptor	*NR3C2*
Retinoic acid receptor α	*RARA*
Retinoic acid receptor ß	*RARB*
Somatostatin	*SST*
Vasopressin	*VAP*
Melanocyte-stimulating hormone	*POMC*
Secretin	*SCT*
Insulin	*INS*
Leptin	*LEP/OB*
Oxytocin receptor	*OXTR*
Follicle stimulating hormone receptor	*FSHR*
Thyroid stimulating hormone receptor	*TSHR*
Insulin-like growth factor receptors	*IGF1R/IGF2R*

31. While the regulation of chromatin organization is part of the mechanism for rapid activation or silencing of gene expression, it was described earlier (**section 2.2**) how the same mediators can propagate their patterns of activity to daughter cells, allowing them to play a role in mediating cellular memory and permanent changes in cellular states such as differentiation or reprogramming. Why chromatin organization proceeds from a dynamic, reversible state to one that is stable and irreversible is not known, although it is likely that this is a common decision within differentiating cells during development. The epigenetic changes we observe associated with diseases may represent these decisions being made in an abnormal manner. One paradigm of note is intrauterine growth restriction (IUGR), which has been observed in humans and mammalian model organisms to increase the risk of the affected individual to develop obesity and type 2 diabetes mellitus in adulthood, which in the case of humans is decades subsequent to the causative environmental event (Simmons 2008). When Thompson et al. (2010) (Thompson, Fazzari et al. 2010) studied a rat model of IUGR and quantified cytosine methylation throughout the genome in beta islet cells from the pancreas of young adult rats, they found a distinct pattern of methylation discriminating the animals that had undergone IUGR, at loci already implicated in glucose metabolism or type 2 diabetes mellitus (Thompson, Fazzari et al. 2010). It has been proposed that IUGR induces an adaptive response to the scarcity of calories *in utero*, causing the foetus to reprogram its metabolism during development to hoard calories, which becomes a maladaptive behavior postnatally in the presence of adequate nutrition (Gluckman and Hanson 2004). This represents a paradigm for a remote event causing epigenetic changes that confer a cellular memory of phenotypic consequence. Such a model of epigenetically-mediated changes conferring cellular memory appears to be worth considering for normal endocrine processes and for abnormal hormonal exposures such as those from endocrine disruptors.

32. There are examples of endocrine disruptors for which effects may be mediated by different epigenetic and transcriptional regulatory processes. The endocrine disruptor bisphenol A (BPA) has been a major focus of investigation for some time, given its broad exposure within the population (Calafat, Ye et al. 2008) and the observed effects in animal models on the development of breast and prostate (reviewed in (Weng, Hsu et al. 2010)). Interestingly, there is little published to demonstrate epigenome-wide effects of bisphenol A, with several reports focusing on individual loci (Dolinoy, Huang et al. 2007; Bromer, Zhou et al. 2010; Weng, Hsu et al. 2010), and two relatively limited genome-wide studies of cytosine methylation in mice, one using Restriction Landmark Genomic Scanning (RLGS) technique on DNA from mouse forebrain (Yaoi, Itoh et al. 2008), the other testing prostate tissue using Methylation-Sensitive Restriction Fingerprinting (MSRF) (Ho, Tang et al. 2006), both of which are based on gel electrophoresis and are relatively limited in their genomic comprehensiveness. Despite this, these studies all showed changes in cytosine methylation associated with exposure, some changes occurring at loci that were found to be transcriptionally altered. While these studies have established a foundation for more detailed and sensitive investigation of effects on cytosine methylation, despite the availability of genome-wide methylation assays for some time (Zilberman and Henikoff 2007) these studies have yet to be published.

33. Exposure to endocrine disruptors other than bisphenol A has also been found to be associated with epigenetic changes. Pregnant rats were exposed to high doses (100-200 mg/kg/day) intraperitoneally of the oestrogenic methoxychlor or the androgenic vinclozolin endocrine disruptors between embryonic days 8-15, and spermatogenesis was observed to be abnormal and compromised in several generations of males subsequently in the absence of subsequent exposures (Anway, Cupp et al. 2005). This transgenerational inheritance of the phenotype suggested an epigenetic mechanism, tested by performing cytosine methylation analyses on testes, again using methylation-sensitive restriction enzymes and a gel electrophoresis detection step. Changes in methylation were indicated by these studies, and while sodium bisulphite validation (see **section 7.4**) was described the primary data were not presented in that original study (Anway, Cupp et al. 2005). While this study has achieved a very high profile and has been extremely provocative, it should be stressed that these findings were subsequently challenged by other groups (reviewed in (Renner 2009)). In one subsequent study the authors used an oral administration of

vinclozolin to pregnant Wistar rats on days 6-15 *post coitum*, but failed to find the spermatogenesis phenotype in even the F1 generation animals, concluding that anti-androgenic effects must occur in the later (days 16-20) stage of pregnancy (Schneider, Kaufmann et al. 2008). These authors also noted that the no observable adverse effect level (NOAEL) in this study was >100 mg/(kg day) whereas the World Health Organisation's current acceptable daily intake of vinclozolin was 10 µg/(kg body weight day), and the original Anway study used 100-200 mg/(kg body weight day). A second study exactly recapitulated the dosage and route of administration as the original study but also failed to find either a spermatogenesis or a DNA methylation consequence of the vinclozolin exposure (Inawaka, Kawabe et al. 2009). What remains an open question is whether the genetic background of the animals exposed is a critical factor, the original study only seeing this effect in outbred rats (Renner 2009).

34. Another study of methoxychlor exposure in rats used a methylation-sensitive restriction enzyme and gel electrophoresis approach with bisulphite PCR or sequencing to assess methylation at a few loci, finding modest changes in methylation levels (Zama and Uzumcu 2009). Vinclozolin administered to mice allowed testing of the methylation status of several loci undergoing genomic imprinting (at which the paternal and maternal chromosomes have different epigenetic organization that results in parent of origin-dependent gene expression (Kacem and Feil 2009). Bisulphite pyrosequencing at these loci showed very modest degrees of change of cytosine methylation (at most ~20%) associated with exposure (Stouder and Paoloni-Giacobino 2010). The anti-androgenic di-2-(ethylhexyl) phthalate administered to gravid mice results in testicular function abnormalities in offspring, prompting testing of cytosine methylation for overall cytosine methylation levels using high-performance liquid chromatography and DNA methyltransferase expression studies. A global increase in cytosine methylation was observed in the exposed animals, with increases in DNA methyltransferase gene expression and protein levels (Wu, Zhu et al. 2010). No locus-specific studies were performed in this project. Another study by this group showed that the same agent was associated with changes in DNA methylation inconsistently through post-natal life (Wu, Zhu et al. 2010), adding another level of complexity to the variation observed in epigenetic responses to endocrine disruption.

35. There has recently been published a report that links exposure to diethylstilbestrol or bisphenol A with increased expression of *EZH2*, a histone methyltransferase that generates the H3K27me3 repressive modification, when tested in MCF7 breast cancer cells *in vitro* (Doherty, Bromer et al. 2010). The same study tested *in utero* exposure to these agents with the outcome of EZH2 expression in adult mammary gland in a mouse model, finding that both chemicals increased EZH2 protein levels and activity. No locus-specific studies were performed as part of this project, but the next logical step will be to perform H3K27me3 ChIP-seq to see whether this increased EZH2 activity results in new sites of repressive chromatin modifications.

36. As stressed earlier (**section 2.2**), epigenetic regulatory mechanisms are very numerous, and a focus solely on cytosine methylation is unlikely to be sensitive to all changes occurring in response to endocrine disruptor exposure. *In vitro* exposure of mammary epithelial cells to diethylstilbestrol was associated with changes in expression of microRNAs (Hsu, Deatherage et al. 2009). There have yet to be published any studies using genome-wide chromatin immunoprecipitation approaches, and while chromatin looping studies have been employed to test how oestrogen mediates its effects using cultured cells (Hsu, Deatherage et al. 2009), no comparable experiments have been described for endocrine disruptors.

5.0 Assay methods

5.1 Issues to address when considering an epigenomics study

37. The studies described above include several approaches towards assessing the role of epigenetic dysregulation. The simplest approach is to perform a candidate gene study, in which one or more genes

are chosen based on prior suspicion that they may be involved in the cellular phenotype, and epigenetic studies are performed usually targeting the transcriptional start site (promoter) of the gene. Candidate genes are frequently chosen based on their functional properties or because they were found to change transcriptional levels by using gene expression microarrays. The advantages of this kind of approach are those of time and cost, and usually allow highly-quantitative approaches to be performed, at the expense of comprehensiveness and unbiased discovery.

38. As the comprehensiveness and quantitative capabilities of genome-wide assays improved while costs decreased, the focus has shifted towards what can be described as epigenome-wide association studies. Just as genome-wide association studies look for polymorphisms of DNA sequence that are non-randomly associated with disease phenotypes, epigenome-wide association studies aim to discover loci with changes in epigenetic regulation that occur preferentially in subjects with disease.

39. Technical approaches used for epigenome-wide association studies currently include those based on microarrays or on massively-parallel sequencing (reviewed in (Boyle and Furey 2009)), largely superseding the gel-based detection systems described in the prior section. The field of endocrine disruptor biology could benefit from carefully-designed analyses of the epigenome using these updated approaches, especially in human subjects, so it is worth describing some of the challenges involved in performing these studies stringently:

- 40. **Study design**: The fundamental principles guiding toxicology studies, including e.g. relevant dose selection, route of administration and duration of exposure, need to be taken into consideration in the design and interpretation of studies (LeBaron et al., 2010; Goodman et al., 2011)
- 41. **Cell type:** Epigenetic dysregulation events are believed to be somatic (occurring in a subset of cells in the body/organ) rather than constitutional (occurring in all cells of the body), requiring that the cell type mediating the phenotype be sampled. It is possible that with an exposure event the epigenetic effects may be more widespread, allowing easily-accessible cell types to be sampled as a surrogate, but in general if there is a disease phenotype affecting a specific organ, it is presumed that cells from that organ should be sampled. This becomes a problem in human studies when the cell type is relatively inaccessible, and serves as a justification for the use of rodent or other animal models (**section 7**).
- 42. **Cell purity:** A further issue has to do with cell purity. Admixture of other cell types presents a challenge because the epigenotypes of histologically-distinctive cell types generally appear to be markedly different. If the proportion or type of cell admixture differs systematically between test and control groups, this may exert enough of an influence to confound the experiment, as the effect sizes (discussed below) may be small. Purifying the cells is not without problems either, as it reduces the sample amount to the point that we may not have sufficient starting material for the epigenomic assay.
- 43. **Choice of assay:** It has also been stressed that there are numerous possible regulators of the epigenome, which creates the problem of choice referred to earlier in **section 2.6**, which may be addressed by using an informative subset of chromatin marks (Ernst and Kellis 2010). In practice, studies usually focus on cytosine methylation, largely because the samples are easily prepared as DNA compared with the more complex sample preparation required for chromatin immunoprecipitation-based assays and because of the relative stability of DNA compared with RNA. Cytosine methylation and transcriptional assays are also reasonably quantitative (Suzuki, Jing et al. 2010), whereas genome-wide chromatin immunoprecipitation assays have been described to be able to call the presence or absence of peaks but have not been shown to be able to discriminate intermediate values. This is a major concern limiting the use of chromatin immunoprecipitation, as the emerging literature indicates that in non-cancer disease states the differences in methylation at a locus tend to be moderate, our IUGR study finding values differing by as little as 10-20% (Thompson, Fazzari et al. 2010), and a recent paper testing liver epigenomes of mice whose fathers

were fed with different diets showed a comparable value (Carone, Fauquier et al. 2010). Mechanistically, this is of interest, as cytosine methylation values in an individual cell can be 0% (neither allele), 100% (both alleles) or 50% (one allele methylated), so the only way that there can be a 20% difference in methylation is when a subset of cells in the population changes its methylation status. This highlights how even modest proportions of contaminating cells can cause problems, as mentioned above, and imposes a requirement for assays to be quantitative as well as comprehensive when performing genome-wide studies. Systems biology meta-analyses approaches can help with the prior refinement and cleaning of such data.

- 44. **Powering the study with adequate cohort sizes:** If the effect size is limited, and the assay has a defined quantitative discriminatory capacity, the cohort sizes required for genuinely comprehensive studies can be modeled. It has been determined that the use of the MSCC (Ball, Li et al. 2009) or our HELP-tagging (Suzuki, Jing et al. 2010) assays will require 100 subjects in each of the test and control groups to be fully powered (unpublished data). While this represents substantially fewer subjects than generally required for genome-wide association studies, it greatly exceeds the numbers described in the studies of **section 4**. When amassing the samples, the cohorts should be chosen with care. It is now recognized that DNA sequence polymorphism can influence chromatin organization, causing it to be polymorphic between individuals (Birney, Lieb et al. 2010; Kasowski, Grubert et al. 2010; McDaniell, Lee et al. 2010), with similar effects now also observed for sequence variation affecting DNA methylation (Gertz, Varley et al. 2011). Cytosine methylation also appears to be influenced by age (Fraga, Ballestar et al. 2005; Thompson, Atzmon et al. 2010) and gender (Sarter, Long et al. 2005), combining to require that cohorts should be matched in terms of self-reported ethnicity, age and gender in order to reduce these potential sources of variability (see next paragraph).
- 45. **Performing and interpreting comprehensively genome-wide assays:** The need for comprehensively genome-wide assays arises because of the emerging evidence that epigenetic regulation of gene expression may not be occurring at predictable locations. There is now a substantial amount of information to suggest that *cis*-regulatory sequences in the genome are frequently located far from promoters (Heintzman, Stuart et al. 2007), and that these loci may be preferentially involved in mediating disease states, as found by Thompson et al. (2010) in their IUGR study (Thompson, Fazzari et al. 2010). While microarray-based approaches have had to compromise to focus on pre-defined loci such as promoters or CpG islands (Hoque, Kim et al. 2008; Yamashita, Hosoya et al. 2009), massively-parallel sequencing-based approaches have no such constraints and can survey the entire genome. This gives rise to a problem of interpretability – while changes at a promoter are relatively easy to interpret in terms of likely effect on that gene's expression, the non-promoter changes may not even be regulating the nearest gene. It is hoped that the functional annotation of mammalian and model organism genomes being undertaken by the ENCODE and Roadmap in Epigenomics projects will provide some insights that will increase the interpretability of many of these loci, but in the interim many studies will generate significant loci in terms of disease associations without insight into how they may be having mechanistic effects. It is for this reason that concurrent transcriptional studies performed on the same samples offer a means of interpreting how an epigenetic regulatory change may be having functional consequences.

46. The understanding and interpretation of perceived epigenetic alterations is complicated by an incomplete understanding of the normal state and dynamic variation of the epigenome, which can differ widely between cell and tissue types and stage of development or age (Le Baron et al., 2010). It is thus important to determine the reference epigenome and its range of variability in each model. In the absence of information regarding the "control" epigenome, any comparison may lead to overestimation or underestimation of the extent of effects of endocrine disruptors, or any other environmental chemicals. For example, Christensen et al. (2009) demonstrated that in normal humans there are changes in the genomic methylation status with age, and whether the location of the target CpG site is in CpG island or outside the island. Loci in CpG islands gained methylation

with age, whereas loci not in CpG islands lost methylation with age, and this pattern was consistent across tissues. In another study, De Bustos et al. (2009) reported that there exists gross regional difference in methylation between tissues from the same individual. However, profiles of the same tissue from different donors were found to be strikingly similar, as well as the profiles of different lobes of the brain. Tissue differences in receptor and enzyme expression are well documented for human and rodent species in the literature. Thus, large epigenetic changes occur in tissues that appear to be normal, and the relationship of these changes to companion genetic changes is of interest to study in the future.

- 47. **Costs:** An impediment to these genome-wide studies has been costs, especially when the cohort sizes of several hundred individuals are required, and massively-parallel sequencing is employed. What is making these assays more affordable at present is the huge amount of sequence now being generated by massively-parallel sequencing, allowing many samples to be combined following barcoding of the individual samples using short sequence tags introduced during library preparation. This multiplexing of samples is driving costs down significantly, to the point that library preparation costs represent the major financial obstacle. With continued increases in sequencing performance, it should be anticipated that these massively-parallel sequencing-based assays will become increasingly cost-effective and will allow their widespread adoption for epigenome-wide association studies.

5.2 Designing an epigenome-wide association study of endocrine disruptors

48. The discussions of **section 5** give some guidelines about how we might go about searching for the effects of endocrine disruptors on the epigenome. As a first step there would be a need to determine which cell type to study. The options are more plentiful in animal models, whereas the cell types that represent hormonally-responsive tissues in humans tend to be difficult to acquire with the exception of spermatozoa, which may allow the effects of anti-androgenic endocrine disruptors to be evaluated. A comparable cell type that could be easily sampled in females to test the effect of anti-oestrogenic agents is not as obvious. Model organisms such as rodents would not have the same constraints, but have other problems with regard to how they reflect human exposure to toxic substances (**section 7**).

49. The next question is which assay to choose. The effects of steroid hormones on the epigenome were summarized in **section 4** and point to numerous chromatin components (nuclear receptors, ligands, enzyme complexes) and modifications (histone acetylation) that are functionally linked to hormonal signaling and would be prime targets for analysis. The drawback of the genome-wide chromatin immunoprecipitation-based assays is their non-quantitative properties, which could be reflected by insensitivity of detection of changes at many loci in the genome where subpopulations of cells alter their epigenetic regulatory patterns. Cytosine methylation has been shown in many of the rodent models to be relatively informative, and genome-wide assays designed to test it are reasonably quantitative, making these a first choice system at present. Some of the strengths and limitations of the assays are summarized in **Table 4**, which focuses on assays based on massively-parallel sequencing, and ranks the assays as first choice and alternative.

Table 4: Molecular epigenomic assay choices in studies of endocrine disruptors.

Molecular mediator	Detection method		Strengths and limitations of method
Primary (recommended)			
DNA methylation	Bisulphite mutagenesis-based	MethylC-seq (Lister, Pelizzola et al. 2009)	Nucleotide resolution, can interrogate most cytosines in genome.

			Expensive
		RRBS (Meissner, Mikkelsen et al. 2008)	Nucleotide resolution, relatively inexpensive Interrogates limited number of cytosines, focused on CpG-dense regions
	Restriction enzyme-based	HELP-tagging (Suzuki, Jing et al. 2010) MSCC (Ball, Li et al. 2009)	Relatively inexpensive, tests CpG-dense and depleted contexts Interrogates limited number of cytosines
	Affinity-based	meDIP-seq (Down, Rakyan et al. 2008)	Can test throughout genome Quantitative capacity limited in CpG-depleted regions, interrogates contiguous groups of CpGs
	Microarray-based	450K Infinium Methylation BeadChip (Illumina) (Bibikova, Barnes et al. 2011)	Inexpensive, design targeted to regions of presumed function Interrogates limited number of cytosines, informativeness depends on design choices
miRNA	miRNA-seq		Quantitative, can identify previously undiscovered miRNAs Library preparation relatively difficult
RNA	RNA-seq (Nagalakshmi, Wang et al. 2008)		Quantitative, can also generate qualitative data about transcription such as alternative splicing Data analysis approaches still being optimized
Secondary (alternative)			
Chromatin post-translational modifications, chromatin constituents	ChIP-seq (Mikkelsen, Ku et al. 2007)		Tests entire sequenced genome Resolution limited, not shown to be quantitative
Chromatin structure	DNase-seq (Song and Crawford 2010)		Identifies important regulatory regions not located at annotated promoters Not shown to be quantitative

50. In addition to the quantitative analysis of the epigenetic regulators themselves, it is valuable to add a transcriptional study of the same cells, so that epigenetic changes can be interpreted in part by presumed effects on gene expression, an especially problematic issue for loci of unknown function.

51. If a preliminary evaluation of the possibility of epigenomic abnormalities is being sought, there are global molecular approaches that could be attempted. Genome-wide cytosine methylation can be tested a number of ways, using high-performance liquid chromatography as described earlier (Zhang, Zhang et al. 2011), testing transposable elements like long or short interspersed nuclear elements (LINEs, SINEs) with bisulphite sequencing (Yang, Estecio et al. 2004), or performing luminometric methylation analysis (LUMA) (Karimi, Johansson et al. 2006), to name a few. If a more functional test is required, the viable yellow (A^{vy}) mouse model has characteristics that have caused it to be described as an 'epigenetic biosensor (Dolinoy 2008). The A^{vy} allele resulted from the insertion of an intracisternal viral A particle (IAP) upstream of the transcription start site of the *Agouti* gene, rendering the coat colour and the body stature of the Agouti mice dependent of the methylation status of the IAP ranging from agouti and obese (yellow, low methylation) to pseudoagouti and lean (brown, high methylation) with several degrees of intermediate mottled phenotypes. The IAP transposable element that alters the coat colour phenotype in these animals appears to be unusually susceptible to influences that alter the epigenome, such as dietary influences in mice exposed to endocrine active substances (Dolinoy, Huang et al. 2007), generating a readout in terms of coat colour which is easily recognizable, and allowing direct analyses of the IAP element in terms of its cytosine methylation as a more quantitative readout (Waterland and Jirtle 2003). However, as describe later in **section 7.3**, the evaluation of these viable yellow mice as a model suitable for toxicology studies has not proven to be encouraging.

52. Cell culture systems represent the mainstay of many of the current studies of endocrine disruptors effects upon the epigenome. Potentially interesting cell culture models include those that allow the *in vivo* architecture of the cell type to be recapitulated, such as that described for breast epithelial cells (Lee, Kenny et al. 2007). A problem with cultured (Meissner, Mikkelsen et al. 2008) and transformed (Wild, Funes et al. 2010) cells is that they tend to be substantially modified in terms of their epigenetic organization compared with primary cells, making them poorly comparable with cells sampled from *in vivo* sources. A further problem is that prolonged culture can change the characteristics of cells, so *in vitro* systems are going to be maximally useful if the culture conditions are kept identical between conditions being compared and over time.

5.3 Is the use of model organisms necessary?

53. The decision-making process above has the effect of directing us towards the use of model organisms, primarily because of sample acquisition issues, but there are other factors to consider. The ability to control and monitor exposures with animal colonies kept in controlled conditions should exceed that possible for human subjects. The potentially confounding effect of genotypic polymorphism can be avoided by using inbred strains, and specific genetic backgrounds can be introduced experimentally. Live animals allow metabolism of agents to other active byproducts that is difficult to achieve using cultured cells. Cells *in vitro* are also prone to changes in their epigenetic patterns with culture (Allegrucci, Wu et al. 2007; Meissner, Mikkelsen et al. 2008). There are thus numerous advantages to the use of animal models.

54. One especially advantageous reason for using animal models is the ability to pursue the transgenerational effects of endocrine disruptors (Anway, Cupp et al. 2005; Anway and Skinner 2006; Crews, Gore et al. 2007; Guerrero-Bosagna and Skinner 2009). This has already proven interesting in terms of studies of epigenetic organization in testes (Anway, Cupp et al. 2005), and appears worth pursuing further. It is interesting that cytosine methylation changes have been observed in rat testes occurring sufficiently markedly and reproducibly that they could be detected as a specific effect of vinclozolin (Anway, Cupp et al. 2005). This is a counter-intuitive result given what is known about cytosine methylation during spermatogenesis and development. The vinclozolin-induced changes in methylation would have to survive two massive waves of demethylation of DNA genome-wide, one occurring early during spermatogenesis (at the foetal stage, following the differentiation of primordial

germ cells into early spermatogenic cells), and a second demethylation wave early in embryogenesis that affects the paternally (sperm)-derived haploid genome prior to the maternally-derived contribution, with two phases of remethylation during later spermatogenesis and at the time of implantation (Reik, Dean et al. 2001). There is precedent for epigenetic marks surviving these waves of global cytosine methylation changes, as imprinted loci appear to retain the memory of their gametic origin despite the early post-fertilisation demethylation event (Reik, Dean et al. 2001).

55. So while rodent models have limitations in how they represent human exposures (Stokes 2004), there remain many rodent models in use to test endocrine disruptors, possibly allowing some short-term experiments to be performed to resolve fundamental questions about whether and how endocrine disruptors influence epigenomic organization *in vivo,* whilst not increasing animal usage. The goal should be to limit animal use to the unavoidable transgenerational effects studies, which cannot be reproduced or predicted *in vitro* in cultured cells.

56. Another type of model organism that has great potential in terms of modeling developmental and epigenetic effects on vertebrates and not only aquatic wildlife, is the zebrafish (*Danio rerio*) embryo epigenetic toxicity assay. It is important to note that this is not the same test as the Zebrafish Embryo Toxicity Test (ZFET), currently validated to test acute lethality in fish (OECD, 2011). Zebrafish generation time is rapid and the transgenic lines established so far have been shown to be stable through multiple generations (Udvadia and Linney 2003). Their maintenance is relatively cheap and they are amenable to higher throughput testing. In addition, their use is very much in keeping with the 3Rs, as it is not considered as an *in vivo* test. The zebrafish embryo is establishing itself as a valuable model for toxicity testing, especially for developmental toxicity (Augustine-Rauch, Zhang et al. 2010; McCollum, Ducharme et al. 2011), including that mediated by the ER (Celeghin, Benato et al. 2011). The fish ER has close species similarity to that mammalian counterparts including human ER (Dang 2010). Indeed, the US EPA has developed an expert system based upon fish liver ER (Schmieder, Tapper et al. 2004), and the maternal estrogen receptor 2a mRNA has been shown to affect embryo transcript contents and larval development (Celeghin, Benato et al. 2011). Another receptor, the Retinoic Acid Receptor (RAR), an important heterodimerisation partner also involved in the steroidogenic pathway, has been hypothesized to provide an epigenetic mechanism for initiating the diversification of cell types in the developing zebrafish embryo (Linney, Donerly et al. 2011).

57. Endocrine related and xenobiotic receptors (such as the pregnane X receptor) that have greater species differences would not be such an appropriate mammalian/human model, but the ER would be a good starting point. The vertebrate model organism zebrafish has a reasonably well-studied epigenome that includes DNA methylation as a regulatory process (Wu, Zhang et al. 2011) (unlike other model organisms such as *Drosophila melanogaster* in which DNA methylation is at most minimal (Krauss and Reuter 2011)) and has well-established techniques for chromatin studies (Lindeman, Vogt-Kielland et al. 2009). In addition, studies of miRNA functions in the zebrafish also highlight several common principles underlying the functions of animal miRNAs (Mishima, 2011). The use of zebrafish for studies of endocrine active substance, reproductive and developmental screening is now well-established (reviewed in (Krauss and Reuter 2011), there are a number of suitable lines (Uddvadia and Linney 2003) that could be carefully evaluated to select for particular endpoint development, and with a 1.6 billion basepair genome size would require less sequencing for epigenomic assays than a mammalian organism. Zebrafish appear to represent the most suitable model organism available for studies replicating the effects of endocrine active substances and endocrine disruptors on vertebrates and aquatic wildlife and a model that also has great potential for rapid, reliable and less expensive exploration of the role of epigenetics, aging, senescence and cancer outcomes in relation to endocrine endpoints, an area that is increasingly important to address in regulatory toxicology, but the current higher level (level 5) *in vivo* TGs are unable to address for reasons of high cost, extended time, and the humane concerns related to the extension of such tests.

58. There are clearly well supported reasons for further development in the very near future of the developed model that has been successfully by many laboratories. It could be developed specifically to address epigenetic endpoints in relation to endocrine activity and phenotypic consequences in the model, to assess the quantitative and predictive capacities for later adverse outcomes. It might also be a useful model to assist in the discussion on the treatment of functional genomics in Test Guidelines.

5.4 What are the potential future advances facilitating new approaches?

59. There is reason for optimism regarding our ability to use technology more effectively to gain insights to *the epigen*omic effects of endocrine disruptors. This is largely based on the phenomenal pace at which massively-parallel sequencing is advancing in terms of increasing throughput and reducing costs, exceeding the Moore's law paradigm for the number of transistors that can be placed on an integrated circuit doubling every 2 years – recent experience suggests that sequen*cing cost*s per basepair are dropping at a substantially faster rate (Sboner, Mu et al. 2011). This is going to make cytosine methylation assays more cost-effective, as mentioned earlier, but also more quantitative, as we can move from limited sampling techniques based on restriction enzymes or reduced genomic representations and instead use shotgun bisulphite sequencing (BS-seq, MethylC-seq (Harris, Wang et al. 2010)), a substantially more powerful approach. Chromatin immunoprecipitation-based assays will not change in terms of resolution but it is possible that for transcription factor studies the extra depth of sequencing may allow more comprehensive data to be generated, whereas histone modification studies do not appear to benefit in the same way from greater depth (Rozowsky, Euskirchen et al. 2009).

60. The second area of advance will be in terms of interpretability of findings, highlighted earlier in terms of the non-promoter findings that we will uncover. We are already beginning to understand that transcribed sequences in the genome behave differently in terms of their epigenetic organization compared with non-transcribed sequences, with specific histone modifications (Vakoc, Mandat et al. 2005) and paradoxically increased cytosine methylation (Ball, Li et al. 2009), requiring that we treat these functionally-defined genomic contexts separately in order to be able to interpret results. A goal for many ongoing studies is to be able to define optimal methods for integrating different types of genome-wide data in order to be able to understand epigenomic and transcriptional regulation as a system, including the influences of DNA sequence polymorphism, advances that will greatly facilitate studies addressing specific questions such as the effect of endocrine disruptors upon the epigenome.

6.0 Challenges

61. These advances do not come without cost. The amount of information generated by these increasingly comprehensive genome-wide assays is becoming the single biggest impediment to gaining insights into the underlying biology (Kahn 2011). The data need to be managed and secured as a first step, as all digital information can be easily lost through hardware failures unless steps are taken to maintain the system and store copies remotely. This amplifies the magnitude of the datasets, but allows data derived from precious samples to be maintained for subsequent analysis.

62. The analysis challenge is also substantial (Sboner, Mu et al. 2011). Many analyses cannot be performed using standard desktop computing resources because the processing and storage requirements greatly exceed what they can provide. The analyses of these data require multiple steps, for each of which there are competing analytical approaches rather than universally-accepted standard algorithms. Likewise, quality assessment and control metrics are also heterogeneous and require substantial computational processing to generate meaningful results. The transformation of raw data (microarray fluorescence intensities, massively-parallel sequencing DNA sequence reads) into biological information allows very large initial datasets to be shrunk to relatively smaller and more manageable formats, but then

the next challenge emerges of making sense of this information. Whether this involves comparing the results from that sample with those in a similar and a comparison cohort, or comparing the results against other genomic annotations, the end result is similar in that there need to be multiple datasets assembled in a single analysis. Again, this represents a computational challenge that is usually addressed by high-performance computing resources, with cloud computing as an emerging alternative or complement.

63. When integrating datasets, it becomes necessary to remove nuances about the data and transform information into genomic 'objects' (a categorization of epigenetic events by locus). It is not always apparent that the decisions made about how these kinds of transformations are performed reflect relatively subjective decisions, and these may not always be transparent or well-founded. For example, it was earlier described that increased sequencing of chromatin immunoprecipitation of STAT1 defines increasing numbers of binding sites (Rozowsky, Euskirchen et al. 2009), so the definition of the genomic objects of STAT1 binding sites is dependent upon the depth of sequencing performed, which may not be consistent from sample to sample or lab to lab. This issue reinforces the need for not only breadth in epigenomic studies (genome-wide comprehensiveness) but also depth (at specific loci). Another problem has to do with the assumption that different epigenomic events occurring at the same locus must mean that they are present in the same cell. This can only be confidently concluded when all or the vast majority of the cells have the epigenomic event, requiring in turn a quantitative capability for the assay used. If 50% of the cells are found to have cytosine methylation and 50% of the cells are found to have a specific histone modification at the same locus, it could be interpreted that the same cells have both events occurring, but it is also possible that none of the cells have the same event and that they are mutually exclusive. These are challenges inherent to epigenomic data integration that remain largely unsolved and will need to be a focus for the near future.

7.0 Conclusions and testing recommendations

7.1 Conclusions

64. In conclusion, it is possible to state that the evidence thus far is highly-suggestive of a role for epigenomic dysregulation mediating the effects of exposures to endocrine disruptors. Mechanistically, it is plausible that the epigenome is responsible for some of the phenotypic consequences of these exposures. These conclusions need to be weighed against *the re*lative weakness of the studies performed to date, which have neither been comprehensive nor quantitative, have frequently used *in vitro* tissue culture systems or have used mixed cell types from rodent models. There is a major paucity of human subject data at present, another reason for concern.

65. There are reasons for optimism regarding our abilities to perform well-designed, comprehensive and sensitive studies to test for epigenomic dysregulation following endocrine disruptor exposure. The absence of standardized assays and analytical approaches coupled with the challenges of managing and analyzing data represent impediments to progress, while we also recognize that there are drawbacks to *in vitro* cell culture systems, animal models and human studies, making no system ideal for these studies, although rodent models offer a lot of advantages in the short-term.

7.2 Testing recommendations

66. In defining some testing recommendations, a number of influences are taken into account. Firstly, the need to minimize animal use to the greatest extent possible, so cell culture systems that may be useful are included. Secondly, the need to be guided by prior experience in this field, including dosage regimens for *in vitro* and *in vivo* systems. Therefore a varied range of studies of endocrine disruptor use with epigenetic consequences has been summarized; these studies provided details that could be used to guide formal regulatory pre validation and validation study designs. Only one of the studies (Hsu et al., 2009)

included epigenomic assays, Doherty et al studied EZH2 expression, Chaturvedi et al studied expression levels of a few genes, the Hsu reports studied miRNA and more general transcription, methylation and chromatin studies. The *in vitro* cell systems are listed in **Table 5** and *in vivo* assays in **Table 6**.

Table 5: Published cell culture systems that could potentially be used for testing the epigenetic effects of endocrine disruptors.

Cell type	Agent	Dosage and schedule	Vehicle	Reference
MCF-7	DES	$5x10^{-6}$ - $5x10^{-8}$ M for 48 hours	DMSO	(Doherty, Bromer et al. 2010)
	BPA	$2.5x10^{-5}$ - $2.5x10^{-6}$ M for 48 hours		
CV-1	DHT	10^{-8} M for 24 hours	DMSO:ethanol 1:1	(Chaturvedi, Kumar et al. 2010)
	BCH DDT 2,4'-DDT 4,4'-DDT 2,4'-DDE 4,4'-DDE Procymidone Fenitrothion Vinclozolin Nitrofen Linuron Methoxychlor Difenoconazole Chlozolinate Metribuzin Tetramethrin	10^{-6} M for 24 hours		
Human mammospheres	17ß-estradiol	70 nM for 3 weeks	DMSO	(Hsu, Deatherage et al. 2009; Hsu, Hsu et al. 2010)

DES: Diethylstilbestrol
DMSO: Dimethylsulfoxide
BPA: Bisphenol A
DHT: Dihydrotestosterone
BCH: Brominated flame retardant
DDT: Dichlorodiphenyltrichloroethane
DDE: Dichlorodiphenyldichloroethylene

Table 6: Published animal systems that could potentially be used for testing the epigenetic effects of endocrine active substances.

Animal species and strain	Agent	Dosage and schedule	Vehicle	Tissue sampled, timing	Test Outcome	Comments related to potential EAS-epigenetics test development	Reference
Rat (Sprague-Dawley)	DEX	50 mg/kg per dose PO on: PND 25, 60 or 65	Corn oil	Liver Up to 4 days following exposure	ChIP of PXR at *Cyp3a1* promoter	Major species differences between rat/human PXR and CYP metabolism of DEX, Model more appropriate for drug interaction and species differences in metabolism than EAS.	(Ronis, Chen et al. 2011)
Mouse (Swiss albino)	DDT	50 mg/kg/day in olive oil x 7 days PND 28-42, male	Olive oil	Liver Testis 1 day following last exposure	AR and PXR transcription and subcellular dynamics		(Chaturvedi, Kumar et al. 2010)
Rat (Fisher)	Methoxychlor Vinclozolin	100 or 200 mg/kg/day IP GD 8-15	DMSO	Testis Sperm PND 60 (F1-F4)	Qualitative assessment of spermatogenesis, DNA methylation studies of sperm.		(Anway, Cupp et al. 2005)
Mouse (*a/a*)	BPA	50 mg/kg/day PO 2 weeks pre-mating, then throughout gestation and lactation Adult female	None	Tail Brain Liver Kidney PND 22	DNA methylation studies in tail, brain, liver and kidney	This model is not highly recommended due to the excessive numbers of animals needed for such studies and the qualitative nature of the data obtained makes it an impractical choice.	(Dolinoy, Huang et al. 2007)
Mouse (*a/a*)	Genistein	250 mg/kg/day, 2 week pre-mating then throughout gestation and lactation	Corn oil and soy oil	Tail Brain Liver Kidney PND 22	DNA methylation studies in tail, brain, liver and kidney	This model is not highly recommended due to the excessive numbers of animals needed for such studies and the qualitative nature of the data obtained	Dolinoy et al, 2006

		Adult females				makes it an impractical choice.	
Rat (Sprague-Dawley)	BPA	10 µg/kg 0.1 and 2500 µg/kg	Corn oil	Prostate 28 weeks	Prostate histopathology and DNA methylation studies		(Ho, Tang et al. 2006)
	17ß-estradiol 3-benzoate	PND 1, 3, 5 SQ injections					
Mouse (FVB)	Vinclozolin	50 mg/kg/day IP GD 10-18	Corn oil	Motile sperm Tail Liver Skeletal muscle PND 60 (F1 - F2)	Sperm counts and DNA methylation studies		(Stouder and Paoloni-Giacobino 2010)
Mouse (Kunming)	Di-2-(ethylhexyl) phthalate	500 mg/kg/day PO GD 12.5 –19	Corn oil	Testes GD19	Global DNA methylation quantification, expression levels of DNMTs		(Wu, Zhu et al. 2010)
Mouse (CD-1)	DES	5 mg/kg/day IP GD 9-26	Sesame oil	Mammary 6 weeks	EZH2 expression and function studies		(Doherty, Bromer et al. 2010)
	BPA	10 µg/kg/day IP GD 9-26					

DEX: Dexamethasone IP: Intraperitoneally
PO: *Per os*, by mouth BPA: Bisphenol A
PND: Postnatal day DES: Diethylstilbestrol
ChIP: Chromatin immunoprecipitation AR: Androgen receptor
PXR: Pregnane X receptor DNMT: DNA methyltransferase
GD: Gestational day EZH2: Enhancer of zeste homologue 2

67. Note that only one animal study from **Table 6** in which epigenetic assays were performed also included pharmacokinetic analyses, Doherty et al. measuring BPA levels in mice on gestational day 13, at 1, 6, 12 and 18 hours after the last dose of BPA. They found peak levels of BPA of 24.69 ng/mL at 1 hour following administration, when control (vehicle-only) levels were 1.70ng/mL. At 6 hours the BPA levels were 3 ng/mL, and subsequently indistinguishable from background (Doherty, Bromer et al. 2010). It will be necessary to incorporate information from other studies that do not have epigenetic components for their more detailed pharmacokinetic data, such as the recent report from Prins et al. (Prins, Ye et al. 2011) testing BPA doses in Sprague-Dawley rats.

68. Recommendations for testing, based on existing test systems used by the OECD are listed by broad category below. A point worth mentioning is that the essence of epigenomic dysregulation is the potential for longer-term memory of exposure, making a delay between exposure and effect testing desirable, in contrast with many other outcomes that may be sought. Of these, only the extended one-generation reproductive toxicity study (TG 443) is currently designed to include both epigenetic and endocrine endpoints, making it the most immediately suitable for adaptation. The Zebrafish embryo epigenetic toxicity assay is the most relevant alternative test model with which to proceed for regulatory development (**Section 5.2**).

Table 7: OECD Test Guidelines that could potentially be adapted for epigenomic studies of effects of endocrine disruptors.

Type of study	*Test Guidelines (TG)*	*Description*
		• *Zebrafish embryo epigenetic toxicity assay*
General exposure studies	• TG 451 • TG 452 • TG 453	• Carcinogenicity Studies • Chronic Toxicity Studies • Combined Chronic Toxicity/Carcinogenicity Studies
Post-mitotic cell studies	• TG 424	• Neurotoxicity Study in Rodents
Prenatal effects	• TG 414 • TG 426	• Prenatal Development Toxicity Study • Developmental Neurotoxicity Study
Reproductive effects	• TGs 415, 416 • TG 421 • TG 422 • TG 443	• One- and Two-Generation Reproduction Toxicity • Reproduction/Developmental Toxicity Screening Test • Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test • Extended One-Generation Reproductive Toxicity Study
Potentially relevant tests to be used in combination	• TG 473	• *In vitro* Mammalian Chromosome Aberration Test

7.2.1 Choice of test.

69. A modification of TG 473 (*In vitro* Mammalian Chromosome Aberration Test) that left out the use of metaphase-arresting substances in exposed cells could allow these cells to be used to screen for epigenetic effects, without having to introduce a new cell type for study. **Section 7.3** also describes how human embryonic stem cells (hES) may represent a new reagent for *in vitro* cultured cell studies of chemical toxicity, although efforts to explore this do not appear to have been successful so far, for regulatory use, and rodent and zebra fish embryo models have been demonstrated to be more reliable and accurate in test chemical predictions (for teratogenicity (Augustine-Rauch, Zhang et al. 2010)), and thus more amenable to regulatory requirement needs. Where definitive animal tests are necessary, there are three tests that appear suitable for testing epigenetic effects of exposures throughout the body which could be performed by harvesting material from animals sacrificed as part of ongoing studies without the need to test further animals. TGs 451 (Carcinogenicity Studies), 452 (Chronic Toxicity Studies) and 453 (Combined Chronic Toxicity/Carcinogenicity Studies) all involve animal exposures by different routes, with a necropsy subsequent to the exposure schedule that would allow the opportunity for tissue harvesting. Correlative histopathology and clinical chemistry studies will allow some epigenetic findings to be interpreted. The cell types to be tested should meet the criteria of **section 5** in terms of purity and phenotypic relevance, in the current case choosing cells that are hormonally-responsive. TG 424 (Neurotoxicity Study in Rodents) focuses more specifically on the central nervous system, composed mostly of post-mitotic cells, with studies of brain function to complement histopathology and epigenetic studies. Prenatal effects are potentially studied using TG 414 (Prenatal Development Toxicity Study) which involves the exposure to animals of agents during pregnancy, testing the foetus at term for abnormalities, while TG 426 (Developmental Neurotoxicity Study) allows the offspring to be born and to develop, testing specifically for neurological consequences. Tissues harvested at both timepoints could shed light on epigenetic effects of agents used for exposure.

70. It would be premature to draw any firm conclusions about the application of the threshold (TTC) approach in relation to substances that may have endocrine activity, although there are very few examples of where this has been done for specific endocrine mechanisms (such as ER alpha mediated thresholds in the aquatic environment) and thus this would also currently apply for epigenetic threshold mechanism in relation to the endocrine system. Regarding thresholds for epigenetic changes, whether the effects of endocrine disruptors (or any other chemical) are manifested in a binary fashion beyond a certain threshold exposure dose, such as, an “open” or “closed” state of the chromatin, “hypermethylated” or “hypomethylated” CpGs, or whether epigenomic changes might be dose-dependent is also unclear. However because DNA methylation can be quantitated, DNA hypermethylation or hypomethylation of specific target loci can be used to determine whether epigenomic changes show a dose-dependent response. Thus inducing an epigenetic change by increasing the dose beyond relevant real-life scenario exposure levels, (as is seen in the case of vinclozolin studies) may not be the best way to draw conclusions about the potential, real-life, epigenetic effects of endocrine disruptors. There is an identified research need to examine epigenomic alterations at very low doses, and whether there is any significance.

71. One concern about endocrine disruptors is that they may have lingering transgenerational effects mediated by the epigenome. A genuinely transgenerational study requires looking as far as the F3 generation (Skinner 2008). Current test systems only proceed to the F2 generation, so available mammalian test systems are not going to be definitive in testing for transgenerational effects, and will at best generate indicative, preliminary insights. There exist five tests that may allow such preliminary testing for transgenerational effects mediated by epigenetic dysregulation. TGs 415 and 416 (One- and Two-Generation Reproduction Toxicity), TG 421 (Reproduction/Developmental Toxicity Screening Test) and TG 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) all involve exposures followed by assessments of reproductive capacity and/or gonadal histology and function. Of these, the TGs 415 and 416 (One- and Two-Generation Reproduction Toxicity) could both allow multiple tissues to be sampled in offspring of parents exposed to the agent of

interest, allowing screening for inherited epimutations in these samples. The new Extended One-Generation Reproductive Toxicity Study (TG 443) also has the potential for the necropsy and neurological studies of the tests described in the previous paragraph, and therefore represents a recommended mainstay for animal testing.

7.2.2 Specific examples of tests

72. Cultured cells will be exposed to chemicals to look for toxicological effects on those cells. Epigenomic studies can use the same test parameters (*e.g.* dose levels) as those that led to toxicological effects in previous studies, with the caveat that the cells should be assessed for viability, as the presence of dead cells in substantial proportions (>10%) in the material assayed could cause artefacts, requiring that viable cells be sorted from the dead cells (*e.g.* using propidium iodide and flow sorting). The cells will need to be fixed with formaldehyde soon after harvesting if chromatin immunoprecipitation is a planned assay, whereas flash freezing and/or the use of RNAlater (Qiagen) can be used to preserve RNA for later expression studies. Flash freezing is sufficient for preservation of DNA for later cytosine methylation analyses. Samples should be stored at -20°C until ready for use.

73. Epigenomic assays should initially be performed on at least 10 exposed and 10 non-exposed samples, allowing the presence and degree of epigenomic dysregulation to be assessed, allowing a decision to be made about whether to (a) proceed with the number of further samples defined by the effect sizes and power calculations estimated on the basis of the first groups, (b) perform single-locus validation on loci appearing to be non-randomly altering their epigenetic regulatory patterns in response to chemical exposure, expanding the numbers beyond the initial limited groups.

74. Should animal systems be required, the exposures should be those that (a) reproducibly induce the associated phenotypic effect in that animal and, ideally, (b) are comparable to any exposure described for humans. It may be necessary to perform pharmacokinetic profiling in the animal system if human exposures are described in terms of measured total and free concentrations of the chemicals in blood or other body samples. Human and rodent BPA pharmacokinetic and serum levels have recently been shown to be similar using both oral and sub cutaneous dosing. Free and total BPA at C_{max} were 1.77 and 2.0 ng/ml, respectively following injection and 0.26 and 1.02 ng/ml, respectively following oral exposure. The AUC_{0-2} for free and total BPA was 4.1-fold and 1.8-fold greater, respectively, in s.c. versus oral delivery. While exposure route affected BPA metabolism, internal dosimetry following s.c. injection of 10µg BPA/kg BW is similar to BPA levels observed in humans. (Prins et al 2011) Serum levels of bisphenol A that were measured in cohorts of women with and without histories of recurrent miscarriages, revealed that the former group had mean±SD values of 2.59±5.23 ng/mL with the controls averaging 0.77±0.38 ng/mL (Sugiura-Ogasawara, Ozaki et al. 2005), levels comparable to those that can be generated in mice (Doherty, Bromer et al. 2010). A bisphenol A study could therefore be guided by prior studies (Doherty, Bromer et al. 2010) with a dosage schedule of 5 mg/kg/day IP in sesame oil administered to gravid mice between gestational days 9-16, testing cells from offspring at 6 weeks of age.

75. Cell samples should be collected from (a) the phenotypically-affected organ(s) (b) to a reasonable degree of purity, as discussed in **section 5.1**. For example, motile sperm collected from mice following vinclozolin exposure (Stouder and Paoloni-Giacobino 2010) represents an homogenous cell population manifesting the phenotypic effect of the chemical, meeting these criteria. It is probably worthwhile sampling more cell types than are obviously necessary at the outset, as this will allow future studies to be performed without the need to use more experimental animals.

7.3 Potential new test systems

76. While there are many ways that current OECD test systems can be adapted quite easily for analysis of epigenetic dysregulation, it is worth considering a couple more possibilities that are often suggested. The viable yellow (A^{vy}) mouse model was described earlier (**section 6**), allowing screening for effects of exposures during pregnancy by the use of coat colour or cytosine methylation analysis of the IAP element (Waterland and Jirtle 2003). This is not the only potentially useful mouse resource, however. The Axin 1 fused ($Axin1^{Fu}$) mice also have a visible phenotype that is responsive to influences perturbing the epigenome (Waterland, Dolinoy et al. 2006). Mice have been described that variegate transgenes expressed in peripheral blood, allowing genetic screens to look for mediators of the variegation phenotype (Ashe, Morgan et al. 2008), a system that may be amenable to testing for epigenetic regulatory polymorphism. In each case the animals could be used as a means of screening for epigenetic disturbances, without the need for genome-wide molecular assays at the outset. Set against this is the lack of insight into how sensitive each mouse system is in reporting diverse influences on the epigenome, making it uncertain how valuable these experimental animals are for screening purposes. Furthermore, a study specifically designed to assess whether the A^{vy} mouse model was suitable for testing in toxicology studies demonstrated that the hundreds of animals needed for such studies and the qualitative nature of the data obtained makes it an impractical choice (Rasoulpour, LeBaron et al. 2011).

77. The other major avenue involves the use of embryonic stem (ES) cells that are *in vitro*-differentiated to the germ cell lineage, which is now technically feasible (Rohwedel, Guan et al. 2001), allowing a cell culture model that may be able to recapitulate the effects of *in vivo* exposures. This is potentially a very interesting means of generating human cell types that are normally very difficult to obtain, and is not restricted to the use of germ cells, as many lineages can now be generated from pluripotent ES and induced pluripotent stem (iPS) cells should different cell types be potentially informative. Both human and mouse ES cells can be used in this way, mouse being generally easier to grow and manipulate than human ES cells. The Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS) initiative in Europe (http://www.esnats.eu/) represents a formal attempt to use ES cells for toxicity testing, potentially providing a new system that can be widely adopted and allow more limited use of animal systems. In practice, however it is the mouse ES systems that will be more feasible and reliable to utilise for regulatory testing purposes. The use of these systems participates in the recent progress in understanding the underlying molecular mechanisms of epigenetics in embryonic stem cells as compared to those of differentiated cells and has unveiled key regulatory roles of epigenetic marks driving cellular pluripotency, differentiation and self-renewal/proliferation (see Ang et al., 2011, Barrero et al., 2011).

7.4 Molecular validation of tests

78. The epigenomic tests of greatest current value are those that study cytosine methylation, for reasons described earlier, and will represent the cornerstone of epigenomic testing for some time to come. Other valuable tests will include transcriptional profiling (of RNA and of small processed RNAs) and chromatin immunoprecipitation-based techniques.

79. The validation of each requires a different type of assay. For cytosine methylation, the gold standard is the chemical mutagenesis of DNA with sodium bisulphite to create uracil where there existed an unmethylated cytosine in the original DNA, whereas methylcytosine remains unconverted. Quantitative single locus studies of PCR amplicons that compare the proportion of cytosine to thymine (to which the uracil is converted during PCR) measures the methylation at that locus. Platforms such as Sequenom's MassArray (Ehrich, Nelson et al. 2005) or Qiagen's Pyrosequencer (Fakhrai-Rad, Pourmand et al. 2002) can perform this measurement highly quantitatively.

80. For transcriptional profiling and for chromatin immunoprecipitation, validation is performed by quantitative PCR using primers directed at specific loci. The relative enrichment of one locus compared with another is compared with that predicted from the genome-wide approach as a means of quantitative validation.

81. These validation steps are appropriate for testing how individual experiments perform, but a second avenue of validation is to test how variable are the individual experiments themselves. Validation should seek to capture not only experimental variability but also the variability of the biological system. The former can be assessed by performing replicate experiments repeatedly on the same sample, while the latter is best assessed by testing multiple separate samples. The goal is to determine how much of an influence experimental variability has on biological variability, and how much influence biological variability has on the test system, combining to generate a measure of confidence in the results as a whole. As added measures of stringency, reproducibility in independent laboratories at different times is also essential for confidence in the results.

82. Any identification of epigenomic effects with endogeneous hormones (dihydrotestosterone, 17ß-estradiol) as used previously (Ho, Tang et al. 2006; Chaturvedi, Kumar et al. 2010) would provide a very useful benchmark for candidate endocrine disruptors, which can be substantially less potent in inducing cellular proliferation but comparable in other respects such as inducing calcium influx when compared with endogeneous hormones (vom Saal and Hughes 2005).

83. What is not yet possible is the ability to influence epigenetic regulation at specific loci to make them reflect those observed associated with the phenotype of interest. For example, it is impossible to turn a locus from an unmethylated to a methylated state, although global methylation can be driven in different directions by drugs (Claus and Lubbert 2003; Pogribny, Tryndyak et al. 2008) or diet (Niculescu and Zeisel 2002). Functional validation remains an elusive component of current studies of epigenomic dysregulation.

8.0 Recommendations

84. It is clear that epigenetic modulations underlie critical developmental processes and contribute to determining adult phenotype. Moreover, phenotypic alterations due to exposure to environmental insults during sensitive periods of development are mediated through alterations in epigenetic programming in affected tissues. Consequently, monitoring such marks in response to toxicant exposure may in future provide a valuable tool for predicting adverse outcomes.

85. However, there remains a need for further fundamental research to allow a more robust basis for Test Guideline recommendations. In particular, there is a need to improve knowledge on the links between the modulation of the epigenome and associated phenotypes. In addition, although there is evidence to suggest that epigenomic dysregulation might mediate effects of exposures to endocrine disruptors, it is uncertain as to whether these changes are truly predictive of adverse outcome(s). Results obtained in the OECD transgenerational assays will not directly indicate whether the observed effects occur via an epigenetic mechanism of action. Adverse effects observed in these studies could be used to inform future tests specifically designed to investigate the mechanism of action. Follow up studies should include both an epigenetic, as well as a genomic component to differentiate the contribution of potentially compensatory mechanisms.

86. While it may be premature to initiate OECD Test Guideline activity, because of the rapid scientific development in this field, it is important to monitor progress.

87. It's recommended that, within a few years, an Expert group be convened, in coordination with other relevant OECD groups (Advisory Group on Molecular Screening and Toxicogenomics, group on Adverse Outcome Pathways and Validation Management Groups) to identify promising models to be developed into TG(s).

88. In addition to research exploring the link between epigenetic effects and adverse outcomes, the following issues could be considered to ensure that ongoing research supports future TG development. Promising models, such as those present in Table 8, need to be further explored or developed. **Table 8** indicates how these tests may start to be integrated, and identifies some preliminary reference chemicals to assist with the development of such a battery of tests. Additionally, the identification of prototype chemicals is necessary to determine the sensitivity and specificity of model systems. To understand the linkage between chemically-induced epigenetic modifications and phenotypic outcomes, data on epigenetic endpoints could be obtained from samples collected from *in vivo* models, particularly those adequately sensitive to ED-induced effects across various life stages. This information will be useful to refine testing designs for *in vitro* and *in vivo* test models. An important goal would be to develop *in vitro* and short-term assays for the assessment of chemically-induced epigenetic changes predictive of adverse outcomes, taking into account the role of nuclear receptors as transcriptional factors in the mechanism of action of endocrine active substances.

Table 8: Updated OECD endocrine disruptor testing conceptual framework combined with potential epigenetic tests and preliminary reference chemicals.

Level	**Mammalian and non mammalian Toxicology**	**Epigenetic test information**	**Potential prototype chemicals to determine the sensitivity and specificity of model systems**
1 Existing Data and Non-Test Information	Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability	Epigenetic literature review information	
1	All available (eco)toxicological data from standardized or non-standardized tests.	Epigenetic literature review information	
1	Read across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions	e.g literature-derived information about DNA methylation, RNA and miRNA expression studies and chromatin structure and modification data, with analyses to identify biomarker s for detection of compounds with epigenetic ED activity	
Level 2 *In vitro* assays providing data about selected endocrine mechanism(s)/pathways	Estrogen or androgen receptor binding affinity	Combine with TG 473 but leave out the use of metaphase-arresting substances in exposed cells, this could then be used to screen for epigenetic effects.	**Positive** for ER 17β estradiol **Positive** for ER and epigenetic effects DES BPA Genistein Equol (includes metabolism)

			Positives: for AR Testosterone **Positives:** for AR and epigenetic effects Vinclozolin, Flutamide ,Hydroxyflutamide (metabolite) **Negatives:** for ER effects Corticosterone, Spironolactone, Atrazine, Linuron
2	Estrogen receptor transcriptional activation (TG 455)	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Studies of chromatin components and structure.	**Positives:** for ED and epigenetic effects DES BPA Genistein Equol (includes metabolism) OH Tamoxifen
2	Androgen or *thyroid* transcriptional activation (if/when TGs are available)	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Studies of chromatin components and structure.	**Positives**: for ER and epigenetic effects DES; BPA; Genistein; Equol (includes metabolism) **Positives**: for AR and Epigentic effects Vinclozolin, flutamide,

			hydroxyflutamide (metabolite)
2	Steroidogenesis *in vitro* (draft TG 456)	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Studies of chromatin components and structure. - Multivariate/systems analysis to identify key regulatory factors mediating variability of steroidogenesis on a chemical specific basis.	**Positives** for ED: Prochloraz, Forskolin, Atrazine, Aminoglutethimide, Bisphenol A, DBP **Negative** for ED: human chorionic gonadotropin (HCG)
2	*MCF-7 cell proliferation assays (ER ant/agonist)*	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Studies of chromatin components and structure. - Multivariate/systems analysis to identify what is mediating variability of cell proliferation	*As for ER/transactivation assays, plus substances acting through estrogenic but not receptor pathways (e.g. through non genomic pathways and SULTs, (OP), DBP (dibutylphthalates)).*
2	*Zebrafish embryo epigenetic assay*	**Relevant endpoints**: - DNA modifications (cytosine methylation) - Studies of chromatin components and structure.	
2	*Possible additional examples* *1. Casa assay (sperm cell toxicant* *2. Comet assay (sperm cell*	**Relevant endpoints**:: - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Luminometric methylation analysis (LUMA) for	1. *Valproic acid, DES, lindane, carbenazim, nonylphenol.* *2. DES, lindane, carbenazim, nonylphenol di-2-(ethylhexyl)*

	mutagen) *3. Sertoli cell assay* *4. Leydig cell assay (cross ref with steroidogenesis assay TG 456)* *5, oogenesis, follicular culture* *6. Mouse embryonic stem D3 cell assay (Kleinstreuer et al 2011)* *7. Human embryonic stem cells* *8. Rat whole embryo culture toxicity assay*	global methylation analyses. - Studies of chromatin components and structure. - Multivariate/systems analysis to elucidate relevant regulation factors and pathways	*phthalate, DBP.* *3. BPA and as above* *4. DES, carbenazim, nonylphenol, taxol, ketoconazole.* *5. DES, genistein, carbenazim, nonylphenol, ketoconazole.*
Level 3 *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1]	Uterotrophic assay (TG 440)	**Less relevant endpoint:** correlation changes in uterine tissue with molecular changes (epigenomic assays)	
3	Hershberger assay (TG 441)	No end organ present, not appropriate for testing .	
Level 4 *In vivo* assays providing data on adverse effects on endocrine relevant end-points[2]	Repeated dose 28-day study (TG 407) TG 422	**Relevant endpoints**:**:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies. - Studies of chromatin components and structure. e.g. Testicular histopathology combined with epigenomic dysregulation assays With tissues of interest available, need to consider issues of sample collection and preservation, cellular heterogeneity etc., as discussed in text.	

4	Repeated dose 90-day study (TG 408)		
4	1-generation assay (TG 415)	1. Combination with TGs 451 (Carcinogenicity Studies), 452 (Chronic Toxicity Studies) and 453 (Combined Chronic Toxicity/Carcinogenicity Studies) with focus on hormonally-responsive tissues.: combination with epigenomic assays 2. The rat model of IUGR and quantified cytosine methylation throughout the genome in beta islet cells from the pancreas of young adult rats, results indicate a distinct pattern of methylation discriminating the animals that had undergone IUGR, at loci already implicated in glucose metabolism or type 2 diabetes mellitus (Thompson, Fazzari et al. 2010). BPA studies all showed changes in cytosine methylation associated with exposure, some changes occurring at loci that were found to be transcriptionally altered.	Valproic acid (male: reduction of spermatogenesis, testicular atrophy, degeneration of seminiferous tubules; female: polycystic ovaries high serum testosterone and menstrual disorders. Teratogenic).
4	Prenatal Development Toxicity Study (TG 414)		
4	Chronic toxicity and carcinogenicity studies (TG 451-3)		
4	Reproductive screening test (TG 421 if enhanced)	Oestrus cycles, follicle counts,,oocyte maturation, ovarian integrity; spermatogenesis combination with epigenomic assays, toxicogeonmics and multivariate data analyses	
4	Combined 28 day/reproductive screening assay (TG 422 if enhanced) Developmental Neurotoxicity	Prenatal effects are potentially studied using TG 414 (Prenatal Development Toxicity Study) which involves the exposure to animals of agents during pregnancy, testing the foetus at term for	

	(TG 426)	abnormalities, while TG 426 (Developmental Neurotoxicity Study) allows the offspring to be born and to develop, testing specifically for neurological consequences. Tissues harvested at both timepoints could shed light on epigenetic effects of agents used for exposure.	
Level 5 *In vivo* assays providing *more comprehensive* data on adverse effects on endocrine relevant endpoints over *more extensive parts of the life cycle of the organism*	Extended one-generation reproductive Toxicity Study (TG 443)	Necropsy and neurological studies of the tests for TG 426, 414, 424 etc,	Valproic acid, DES, lindane, carbenazim, nonylphenol BPA, DBP, DEHP, taxol, ketoconazole, genistein, vinclozolin, methoxychlor
5	2-generation assay (TG 416 most recent update)	TG (416) could allow multiple tissues to be sampled in offspring of parents exposed to the agent of interest, allowing screening for inherited epimutations.	DES, lindane, carbenazim, nonylphenol BPA taxol, ketoconazole, genistein, vinclozolin, methoxychlor and as above

Italicised tests are not in OECD TG workplan as yet.

LIST OF ABBREVIATIONS

BCH: a brominated flame retardant

BPA: Bisphenol A

BS-seq, MethylC-seq: Two techniques for shotgun sequencing of bisulphite-converted DNA

CBP: CREB binding protein

CG/CpG: Cytosine-guanine dinucleotide

ChIP: Chromatin Immunoprecipitation

CpG island: a region unusually enriched in CpG dinucleotides

CV-1: a cell line derived from an adult male Cercopithecus aethiops monkey kidney

DDE: Dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

DES: Diethylstilboestrol

DEX: Dexamethasone

DHT: Dihydrotestosterone

DHT: Dihydrotestosterone

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferase

ED: Endocrine disruptor

ENCODE: ENCyclopedia Of DNA Elements

ES: Embryonic Stem

ESNATS: Embryonic Stem cell-based Novel Alternative Testing Strategies

EZH2: enhancer of zeste homolog 2

GD: Gestational day

HELP: HpaII tiny fragment Enrichment by Ligation-mediated PCR

IAP: Intracisternal A Particle

IHEC: International Human Epigenome Project

IP: Intraperitoneally

IUGR: Intrauterine growth restriction

Kg: kilograms

LINE: Long Interspersed Nuclear Element

LUMA: Luminometric Methylation Assay

MCF7: Michigan Cancer Foundation-7 breast cancer cell line

meDIP: Methyl DNA immunoprecipitation

miRNA: micro RNA

mL: millilitres
modENCODE: ENCyclopedia Of DNA Elements for model organisms
MPS: Massively-parallel sequencing
MSCC: Methyl-Sensitive Cut Counting
MSRF: Methylation-Sensitive Restriction Fingerprinting
ng: nanograms
OECD: Organisation for Economic Cooperation and Development
P450scc: P450 cholesterol side-chain cleavage enzyme
PCAF: p300/CBP-Associated Factor
PCR: Polymerase Chain Reaction
PND: Postnatal day
PO: *Per orem*, by mouth
RLGS: Restriction Landmark Genomic Scanning
RNA: Ribonucleic acid
RRBS: Reduced representation bisulphite sequencing
SD: Standard Deviation
SINE: Short Interspersed Nuclear Element
STAT1: signal transducer and activator of transcription 1
TCGA: The Cancer Genome Atlas
TG: Test Guideline
UHRF1: ubiquitin-like with PHD and ring finger domains 1
µg: micrograms

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