Draft 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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**Table of Contents**

1. Introduction ......................................................................................................................... 1-5

2. The Corticotropin-Releasing Hormone/ Vasopressin:ACTH:Glucocorticoid Signaling Pathway ................................................................................................................................. 2-1
   2.1 The HPA Axis: Hypothalamic Pituitary Adrenocortical .................................................. 2-1
      2.1.1 Corticotropin-releasing Hormone (CRH) .................................................................. 2-2
      2.1.2 Arginine Vasopressin (AVP)/Arginine Vasotocin (AVT) ....................................... 2-2
      2.1.3 Corticotropin (ACTH) ............................................................................................... 2-3
      2.1.4 Luteinizing Hormone (LH) and Chorionic Gonadotropin (CG) ............................ 2-3
      2.1.5 Glucocorticoid (GCs) .................................................................................................. 2-3
         2.1.5.1 Glucocorticoid Receptors (GRs) ........................................................................... 2-3
         2.1.5.2 CRH and Glucocorticoid-binding Protein (transcortinprotein and glucocorticoid-binding protein [transcortin]) ................................................................. 2-4
      2.1.6. Neuroendocrine Regulation of the HPA Axis ............................................................ 2-4
   2.2 Potential Effects of EDCs on the HPA Axis ...................................................................... 2-4
   2.3 Precedent Chemicals as Potential Disruptors of the HPA Axis ....................................... 2-5
      2.3.1 Steroid Synthesis and Receptor Agonists and Antagonists ..................................... 2-5
      2.3.2 Metals .......................................................................................................................... 2-5
      2.3.3 Neuroactive Chemicals ............................................................................................... 2-6
      2.3.4 Vasopressin Receptor Agonists and Antagonists .................................................... 2-6
      2.3.5 CRH Receptor Antagonists ....................................................................................... 2-6
      2.3.6 Pesticides .................................................................................................................... 2-6
      2.3.7 Arylhydrocarbon Receptor (AhR) Agonists ............................................................... 2-6
      2.3.8 Estrogenic and Androgenic Disruptors on the HPA Axis .......................................... 2-7
   2.4 In-vitro Screening Assays .................................................................................................. 2-7
      2.4.1 Computerized Receptor Assay .................................................................................. 2-7
      2.4.2 Competitive-binding Receptor Assays ...................................................................... 2-7
         2.4.2.1 Cell Culture Systems .......................................................................................... 2-7
      2.4.3 Gene-activation Assays ............................................................................................. 2-8
      2.4.4 In-vivo Screening Assays .......................................................................................... 2-8
         2.4.4.1 Fish ....................................................................................................................... 2-9
         2.4.4.2 Amphibians ......................................................................................................... 2-9
      2.4.5 Challenges and Limitations ....................................................................................... 2-9
         2.4.5.1 In-vitro vs In-vivo Assays .................................................................................. 2-9
         2.4.5.2 Mixtures vs. Single Chemicals ............................................................................ 2-10
         2.4.5.3 Other Parameters to Be Considered .................................................................... 2-10
   3. The Somatotropic Axis ....................................................................................................... 3-1
      3.1 Overview ......................................................................................................................... 3-1
      3.2 Consequences of Disruption by Environmental Chemicals ......................................... 3-3
         3.2.1 Estrogenic Chemicals ............................................................................................... 3-3
         3.2.2 Anti-thyroid Chemicals ........................................................................................... 3-3
         3.2.3 Corticosteroid Stimulants ...................................................................................... 3-3
      3.3 Chemicals that Directly Disrupt the Somatotropic Axis ............................................... 3-4
      3.4 Assays ............................................................................................................................. 3-4
         3.4.1 In-vitro Assays ......................................................................................................... 3-4
         3.4.2 In-vivo Assays ......................................................................................................... 3-4
      3.5 Strengths, Challenges, and Limitations ......................................................................... 3-4
   4. The Retinoid Signaling Pathway ......................................................................................... 4-1
## 4. Overview

4.1 Retinoic Acid Receptor (RAR) Signaling .................................................. 4-1
4.2 The Retinoid X Receptor Signaling Network ........................................... 4-1
4.2.1 Reproduction .................................................................................. 4-2
4.2.2 Development .................................................................................. 4-2
4.2.3 Lipid Homeostasis ......................................................................... 4-2

## 5. Disruption of Retinoid Signaling by Xenobiotics

5.1 Reductions in Retinoid Levels ................................................................. 4-3
5.2 RAR Agonists ..................................................................................... 4-3
5.3 RXR Agonists/Antagonists .................................................................. 4-3

## 6. Consequences of Disrupted Retinoid Signaling

6.1 Assays ................................................................................................. 4-4
6.1.1 Reporter Assays for the Assessment of Ah Receptor Agonists ........ 4-4
6.1.2 Alterations in Retinoid Levels and Metabolism In Vivo ................. 4-5
6.1.3 RAR Agonists/Antagonists (In-vitro Reporter Assays) .................. 4-5
6.1.4 RXR Agonist-Antagonists (In-vitro Reporter Assays) ..................... 4-6
6.1.5 Adipocyte Differentiation Assay ....................................................... 4-6
6.1.6 Microarrays .................................................................................... 4-7
6.1.7 Alterations in Lipid Levels and Metabolism (In vivo) ..................... 4-7

## 5. The Thyroid-Releasing Hormone:Thyroid Stimulating Hormone:Thyroid Hormone Signaling Pathway

5.1 Introduction .......................................................................................... 5-1
5.1.1 Brief Review of the Hypothalamus:Pituitary:Thyroid (HPT Axis) .... 5-1
5.2 Mechanisms and Potential Points of Action for HPT-disrupting Compounds ................................................................. 5-3
5.3 Previous HPT Assays Review by Zoller et al. ..................................... 5-7
5.4 Update on Assays to Detect Disruption of the Thyroid System Across Taxa 2007–2011 ................................................................. 5-8
5.4.1 In Vitro Assays ................................................................................ 5-10
5.4.1.1 Transient Transfection with TRα and TRβ ................................. 5-10
5.4.1.2 Two-hybrid Assays .................................................................. 5-10
5.4.1.3 DNA Binding Assays ................................................................. 5-11
5.4.1.4 Dendritic Arborization .............................................................. 5-12
5.4.1.5 Neurite Extension .................................................................. 5-12
5.4.1.6 Cell Proliferation Assay ............................................................. 5-13
5.4.1.7 Thyroid Peroxidase Inhibition Assay ....................................... 5-14
5.4.1.9 Iodide Uptake Assay ................................................................. 5-14
5.4.1.10 Thyroid Hormone Binding Protein Assays ............................ 5-14
5.4.2 In Vivo Assays ................................................................................ 5-15
5.4.2.1 Modification of Long-term in Vivo Assays ............................... 5-15
5.4.2.2 Additional In Vivo Models ......................................................... 5-16

## 6. The Vitamin D Signaling Pathway

6.1 Overview of the Vitamin D Signaling Pathway .................................. 6-18
6.1.1 Synthesis ....................................................................................... 6-18
6.1.2 Catabolism .................................................................................... 6-19
6.2 Biological Functions of the Vitamin D Signaling Pathway ................. 6-19
6.2.1 Calcium and Skeletal Maintenance ............................................... 6-19
6.2.2 Immune System Function .............................................................. 6-20
6.2.3 Cancer ......................................................................................... 6-21
6.2.4 Neurodevelopment .......................................................................... 6-21
Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors

6.2.5 Cardiac Function ................................................. 6-21
6.3 Vitamin D Disrupting Chemicals and Effects ........................................... 6-21
  6.3.1 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) ........................................ 6-21
  6.3.2 Polychlorinated Biphenyls (PCBs) ........................................... 6-22
  6.3.3 Secondary Bile Acids ........................................... 6-22
  6.3.4 Ethanol ........................................... 6-23
  6.3.5 Lead ........................................... 6-23
6.4 Screening Assays .................................................................................. 6-23
  6.4.1 Serum Vitamin D Levels ........................................... 6-23
  6.4.2 Changes in Expression of Vitamin D-Responsive Gene Products ........ 6-24
  6.4.3 Skeletal Morphology ........................................... 6-24
  6.4.4 Histology ........................................... 6-24
  6.4.5 Immunohistochemistry ........................................... 6-25
6.5 Current Challenges and Limitations ..................................................... 6-25
  6.5.1 Limited Knowledge Regarding Non-mammalian Vertebrates.......... 6-25

7. The Peroxisome Proliferator-Activated Receptor Signaling Pathway ............. 7-1
  7.1 Overview of PPAR Signaling Pathway ........................................... 7-1
    7.1.1 PPARs in Non-Mammalian Species ........................................... 7-1
  7.2 Precedent Chemicals ........................................................................ 7-2
  7.3 Consequences of Disruption .............................................................. 7-5
    7.3.1 PPARα ........................................... 7-5
    7.3.2 PPARβ/δ ........................................... 7-6
    7.3.3 PPARγ ........................................... 7-7
      7.3.3.1 PPAR Disruption in Wildlife ........................................... 7-8
  7.4 In vitro Screening Assays ................................................................... 7-9
    7.4.1 Ligand Binding Assays ........................................... 7-9
    7.4.2 Transactivation Assays ........................................... 7-9
    7.4.3 3T3-L1 Cell Differentiation Assay ........................................... 7-10
  7.5 In Vivo Screening and Testing Assays .................................................. 7-10
    7.5.1 Peroxisome Proliferation ........................................... 7-10
    7.5.2 Lipid Accumulation ........................................... 7-11
    7.5.3 Microarrays ........................................... 7-11
  7.6 Challenges and Limitations ................................................................ 7-11

8. Epigenetics and Endocrine Disruption ..................................................... 8-1
  8.1 Introduction ....................................................................................... 8-1
  8.2 Definitions ......................................................................................... 8-1
    8.2.1 The Epigenome ....................................................... 8-1
    8.2.2 Epigenomic Regulatory Mechanisms ........................................... 8-2
    8.2.3 Influences Exerted by Epigenomic Regulatory Mechanisms ........ 8-3
    8.2.4 Large-Scale Studies of the Epigenome ........................................... 8-4
    8.2.5 Genome-Wide Assays: The Transition from Microarrays to Massively-Parallel Sequencing ........................................... 8-4
    8.2.6 The Problem of Choice in Epigenomic Assays ............................. 8-5
  8.3 Potential Effects ................................................................................. 8-6
  8.4 Evidence For Endocrine Disruption .................................................... 8-7
  8.5 Assay Methods ................................................................................... 8-9
    8.5.1 The Problem of Choice in Epigenomic Assays ............................. 8-9
    8.5.2 Designing an Epigenome-Wide Association Study Of Endocrine Disruptors ....... 8-11
    8.5.3 Is The Use Of Model Organisms Necessary? .............................. 8-12
    8.5.4 What are The Potential Future Advances Facilitating New Approaches? .. 8-13
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6</td>
<td>Challenges</td>
<td>8-13</td>
</tr>
<tr>
<td>8.7</td>
<td>Conclusions and Testing Recommendations</td>
<td>8-14</td>
</tr>
<tr>
<td>8.7.1</td>
<td>Conclusions</td>
<td>8-14</td>
</tr>
<tr>
<td>8.7.2</td>
<td>Testing Recommendations</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.2.1</td>
<td>General Exposure Studies</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.2.2</td>
<td>Post-Mitotic Cell Studies</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.2.3</td>
<td>Pre-Natal Effects</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.2.4</td>
<td>Transgenerational Effects</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.2.5</td>
<td>In-vitro Systems</td>
<td>8-15</td>
</tr>
<tr>
<td>8.7.3</td>
<td>Potential New Test Systems</td>
<td>8-16</td>
</tr>
<tr>
<td>8.7.4</td>
<td>Validation of Tests</td>
<td>8-16</td>
</tr>
<tr>
<td>9.1</td>
<td>Summary and Conclusions</td>
<td>9-1</td>
</tr>
<tr>
<td>9.1.1</td>
<td>Cross Talk Among Signaling Pathways</td>
<td>9-1</td>
</tr>
<tr>
<td>9.1.2</td>
<td>Assays</td>
<td>9-1</td>
</tr>
<tr>
<td>9.1.3</td>
<td>Epigenetics</td>
<td>9-4</td>
</tr>
<tr>
<td>9.2</td>
<td>Prioritization</td>
<td>9-4</td>
</tr>
<tr>
<td>9.2.1</td>
<td>Highest Priority</td>
<td>9-4</td>
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### Acronyms and Abbreviations

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<td>BIAC</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>LCA</td>
<td>lithocholic acid</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LICA</td>
<td>ligand induced complex assay</td>
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<tr>
<td>LUMA</td>
<td>luminometric methylation analysis</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>MBP</td>
<td>mono-n-butyl phthalate</td>
</tr>
<tr>
<td>MC₄R</td>
<td>melanocortin receptor</td>
</tr>
<tr>
<td>MCPA</td>
<td>2-methyl-4-chlorophenoxyacetic acid</td>
</tr>
<tr>
<td>MCT</td>
<td>monocarboxylate transporter</td>
</tr>
<tr>
<td>MCT8</td>
<td>monocarboxylate transporter 8</td>
</tr>
<tr>
<td>ME</td>
<td>malic enzyme</td>
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<tr>
<td>MEHP</td>
<td>mono-2-ethylhexylphthalate</td>
</tr>
<tr>
<td>MIT</td>
<td>mono-iodothyronine</td>
</tr>
<tr>
<td>MMI</td>
<td>methimazole</td>
</tr>
<tr>
<td>MPS</td>
<td>massively-parallel sequencing</td>
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<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
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<tr>
<td>NIS</td>
<td>sodium-iodide symporter</td>
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<tr>
<td>OECD</td>
<td>Organization for Economic Cooperative Development</td>
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<tr>
<td>P₄</td>
<td>progesterone</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating peptide</td>
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<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
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<tr>
<td>PBB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PBPK</td>
<td>physiologically based pharmacokinetic</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCDD</td>
<td>polychlorinated dibenzo-p-dioxin</td>
</tr>
<tr>
<td>PCDF</td>
<td>polychlorinated dibenzofuran</td>
</tr>
<tr>
<td>PFOA</td>
<td>perfluorooctanoic acid</td>
</tr>
<tr>
<td>PFOS</td>
<td>perfluorooctane sulfonate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
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<tr>
<td>pQCT</td>
<td>peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>PRL</td>
<td>prolactin</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>PTU</td>
<td>propylthiouracil</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>qPCR</td>
<td>chain reaction</td>
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<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RIC20</td>
<td>relative inhibitory concentration</td>
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<tr>
<td>RN</td>
<td>raphe nucleus</td>
</tr>
<tr>
<td>RU486</td>
<td>mifepristone</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>SRIF</td>
<td>somatotropin release inhibiting factor</td>
</tr>
<tr>
<td>STAR</td>
<td>steroidogenic acute regulatory protein</td>
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T3  thyroid hormone
T4  thyroid hormone thyroxine
TBBPA  3,30,5,50-tetrabromobisphenol A
TBTO  tributyltin oxide
TCBPA  3,30,5,50-tetrachlorobisphenol A
TCDD  tetrachlorodibenzo-p-dioxin
TCGA  Cancer Genome Atlas
TD  thyroid disruptor
TG  thyroglobulin
TGB  thyroid binding globulin
TIF2  transcriptional intermediary factor
TIQDT  T4 immunofluorescence quantitative disruption test
TMBPA  tetramethylbisphenol A
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  human thyrotropin
TTR  transthyretin
V1aR  vasopressin-1
VDR  vitamin D receptor
WHO  World Health Organization
ZF  zona fasciculata
ZG  zona glomerulosa
ZR  zona reticularis
1. **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 (Thomas, 1984). 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 the primary targets of endocrine disrupting chemicals (EDCs) since 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 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* (Damstra et al., 2002).

2. The OECD initiated a high-priority activity in 1998 to revise existing and to develop new Test Guidelines for the screening and testing of endocrine disrupting chemicals. As a result, several Test Guidelines have been developed or are presently in development. These Test Guidelines have been integrated into the “OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals” (CF). This framework was discussed and revised at an OECD workshop in Copenhagen (OECD, 2010) and by the Endocrine Disrupter Testing and Assessment (EDTA) Advisory Group in 2011. It lists the OECD TGs and standardized test methods available, under development or proposed that can be used to evaluate chemicals for endocrine disruption. It is intended to provide a guide to the tests available which can provide information for endocrine disrupter’s assessment.

3. The test methods included in this Conceptual Framework are specifically designed to identify and characterize hazards of chemicals that interact primarily with the estrogen, androgen and thyroid (E, A and T) hormonal systems and steroidogenesis pathway. 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, one output from the OECD workshop in Copenhagen (OECD, 2010) was a recommendation that a Detailed Review Paper (DRP) should be drafted to evaluate the effects of chemicals on non-EAT endocrine pathways and explore *in vitro* and *in vivo* test methods for additional key signaling systems important for endocrine toxicity (e.g. glucocorticoid receptors, AhR, peroxisome proliferator-activated receptors (PPARs), and other endocrine related nuclear receptors) to be considered for incorporation into existing or as new OECD TGs. 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. These latter pathways 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.

4. In 2007, the National Research Council published *Toxicity Testing in the 21st Century: A Vision and a Strategy* (National Research Council, 2007). 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. 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; and in vivo assays that would provide a more holistic evaluation of the chemical effects on endocrine signaling processes.

5. The nuclear receptor family has 48 functionally distinct members in humans (Robinson-Rechavi Carpentier et al., 2001). 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 (Thomas, 1984), and this document will address the pathways to which these hormones and receptors contribute.

6. Members of the nuclear receptor family all share a common domain structure. 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. Domain Structure of Hormone Nuclear Receptors

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; therefore, assays for the detection of such disruption will be described in this document.

7. A single neuro-endocrine pathway can influence multiple physiological processes. Accordingly, disruption of one target by an EDC can elicit several diverse apical effects. For example, estrogen signaling is well recognized for its role in female reproductive maturation and function. However, estrogen signaling is also involved in the regulation of glucose tolerance and weight gain (Newbold et al., 2009). Thus, environmental estrogens also have the potential to elicit effects associated with metabolic disease.

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 (National Research
Council, 2007) and for the assessment of physiological consequences. This document is not all inclusive of neuro-endocrine pathways or the physiology processes regulated by the pathways. Rather, the document covers those neuro-endocrine pathways for which significant evidence of susceptibility to disruption exists and assay procedures are sufficiently developed for protocol standardization and validation. Chemicals that are known to disrupt each pathway are described in the respective sections. These chemicals may serve as reference compounds in future standardization and validation of the assays. These pathways are diagrammed in Figure 1-2.

9. Section 8 of the DRP delves into the role of epigenetics in endocrine regulation. Epigenetic modification of the genome provides a means by which endocrine disruption during a window of susceptibility (e.g., neonatal exposure) can result in altered function at a later stage in the life of an organism. The epigenetic modification of germ cells in response to EDCs also provides a potential mechanism for transgenerational effects of such chemicals.

10. The overall intent of this DRP is to provide guidance on testing approaches that can be used for assessing the actions and toxicity of chemicals on neuro-endocrine pathways not addressed in current Test Guides. This DRP is not intended 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. Accordingly, some redundancy in assay descriptions was warranted to maintain the integrity of individual pathways.

![Figure 1-2. Neuro-Endocrine Pathways Known to Be Affected by EDCs Resulting in Symptoms of Metabolic Syndrome and Disruptions in Reproduction, Growth, and Development](image-url)

Arrows denote the direction of the signal.
2. The Corticotropin-Releasing Hormone/Vasopressin:ACTH:Glucocorticoid Signaling Pathway

2.1 The HPA Axis: Hypothalamic Pituitary Adrenocortical

11. The organization and operation of the vertebrate hypothalamus-pituitary-adrenocortical (HPA) axis and its regulation has been the subject of many detailed reviews (e.g., Charmandari et al., 2005; Charmandari, Tsigos, & Chrousos, 2005; Herman et al., 2005; Norris, 2007; Papadimitriou and Prifis, 2009), and only a brief summary is provided here. The HPA axis of vertebrates is primarily a regulator of metabolism (Kyrrou and Tsigos, 2009). It also has and stimulatory and inhibitory effects on the immune system (Dhabhar, 2009), growth (Charmandari et al., 2005), and reproduction in vertebrates and is essential for the birth process in at least some mammals (Kalantaridou et al., 2010; Young et al., 2011). Many aspects of early development, as well as the timing of important events such as puberty and reproductive organ development, are regulated by glucocorticoids (GCs) from the adrenal cortical tissue in all vertebrate groups (Wada, 2008). 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 (see Melmed et al., 2011).

12. 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 GCs, i.e., steroids produced in response to ACTH by the cells of the zona fasciculata (ZF) in the adrenal cortex. The principal GC of primates and bony fishes is cortisol (F), whereas most other vertebrates secrete primarily corticosterone (B). Elasmobranch fish produce a unique GC, 1α-hydroxyxycorticosterone. 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 [E2], and estriol [E3]) from adrenal androgens under the influence of CRH (Young et al., 2011). The synthesis and release of GCs depends upon ACTH and GC 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 [NE]), dopaminergic (dopamine [DA]), serotonergic (serotonin = 5-hydroxytryptamine, 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).

13. The organization and regulation of the HPA axis and the roles of GCs appear to be very similar in all vertebrate groups, although the typical mammalian zonation is generally absent in fish (Norris and Hobs, 2006), amphibians (Heatwole, 2005), reptiles (Tokarz and Summers, 2011), and birds (Wingfield, 2005). For example, AVP is replaced by arginine vasotocin in non-mammalian vertebrates, although the distributions of AVP and arginine vasotocin (AVT) within the brain are very similar (see Moore and Lowry, 1998). 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.

14. 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 of tetrapod vertebrates. Additionally, excess GCs also can influence Na+/K+ balance through the mineralocorticoid receptor (GR). 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 F (Norris and Hobbs, 2006) instead of aldosterone. Possible disruption of aldosterone secretion and consequences for Na+/K+ balance are not considered in this section.

2.1 Corticotropin-releasing Hormone (CRH)

15. 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, and 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 (Kinsey-Jones et al., 2010). During pregnancy, placental CRH is instrumental in controlling fetal HPA functions as well as initiation of birth at least in primates and sheep (Young et al., 2011).

16. 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 (Denver, 2009). CRH also may have direct behavioral actions as a consequence of its actions in other brain regions of vertebrates (Lowry and Moore, 2006; Maier and Watkins, 2005).

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

17. 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.

18. In non-mammalian vertebrates, this role for AVP on ACTH secretion is assumed by AVT (20-22) where AVT binds to AT2 receptors. In amphibians, AVT receptors also are found on adrenocortical cells of Xenopus, and AVT, as well as PACAP stimulates synthesis of B in frogs and salamanders.

19. Magnocellular neurons of the PVN, as well as in the supraoptic nucleus (SON), secrete AVP and send their axons to the pars nervosa, 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\(_{1a}\) and V\(_2\) receptors and are unrelated to the functioning of the HPA axis. Nevertheless, agents that affect AVP levels in the general circulation (see Section C.4, below) might also alter AVP release from the PVN into the HHPS.
2.1.3 Corticotropin (ACTH)

20. Pituitary ACTH is a polypeptide synthesized and released in all vertebrates by pituitary corticotropes under the influence of CRH working through CRH-R1 (Childs, 1992; Mason et al., 2002). In mammals, release of ACTH is enhanced by AVP via binding to V1aR and by AVT binding to AT2 receptors in non-mammals. Corticotropes may also produce another AVP receptor, V2R, 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 (see Kageyama and Suda, 2009).

21. In mammals, ACTH binds to melanocortin receptors (MC3-R) on ZF and ZR cells of the adrenal cortex causing increased cAMP synthesis that, in turn, brings about secretion of GCs and adrenal androgens (DHEA, DHEAS, AND), respectively. Excessive ACTH secretion, as occurs when GCs 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)

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

2.1.5 Glucocorticoid (GCs)

23. Adrenocortical cells are capable of synthesizing GCs and androgens, primarily from cholesterol via progesterone (P4). Hence, interference with the synthesis of P4 from cholesterol or with P4 metabolism, can have repercussions on the ability to synthesize GCs and sex steroids. Some important enzymes for GC synthesis are 3β-hydroxysteroid dehydrogenase (3β-HSD), 11β-hydroxylase (P45011β1, CYP11B1), and 21-hydroxylase (P450c21, CYP21A1). In addition, sulfotransferase 2A1 is necessary for production of DHEAS and aromatase (P450aro, CYP19) for estrogens. The steroidogenic acute regulatory protein (StAR) is required to transport cholesterol to the inner mitochondrial membrane, where the first step in P4 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)

24. Receptors for GCs 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 (GR1) and type 2 (GR2). GCs bind readily to both GRs, but aldosterone, the principle mineralocorticoid produced by the adrenals of tetrapods, binds only to GR1, which often is termed the mineralocorticoid receptor (MR), with GR2 being called simply the glucocorticoid receptor (GR). The receptors in GC target cells are typically GR2 with the exception of the CA-1 neurons of the hypothalamus, where activation of GR1 is involved in GC negative feedback to the HPA axis.

25. Both hyper- and hypoadrenalism frequently are treated with pharmaceuticals. Synthetic GCs, such as prednisone, methyl predisolone, 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 P45011β1 and blocks conversion of P4 to GCs.
and may thus enhance production of adrenal sex steroids. Recently, the presence of another GC receptor, GRβ, has been documented (Kino et al., 2009). However, its physiological role(s) is(are) not confirmed at this time.

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

26. 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. Thus, CRH-BP is an important regulator of plasma CRH in both fetal animals and adults (Young et al., 2011).

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

2.1.6. Neuroendocrine Regulation of the HPA Axis

28. Secretion of CRH is strongly influenced by a variety of neurotransmitters from other brain regions. For example, extrahypothalamic neurons secreting DA from the medial zona incerta of the subthalamus, 5-HT from the raphe nucleus (RN), or NE from the locus caeulesus (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 (Choi et al., 2007).

29. Negative feedback by GCs occurs through CA-1 neurons (GR₁) of the hippocampus, as well as via CRH neurons of the PVN and in pituitary corticotropes (GR₂). 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 GCs during prolonged stress in spite of negative feedback by elevated GCs. 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 Potential Effects of EDCs on the HPA Axis

30. 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. HPA 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 GCs, 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 GC target cells (e.g., cAMP production, Ca²⁺ availability, IP₃ 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 (see Sections 3 through 7 of this volume).

31. From clinical studies of humans (Melmed et al., 2011), we have learned that alterations in the HPA axis can influence the stress response and osmotic balance. GCs 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 GC or GC-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

32. 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 systems with which it interacts, make it a prime target for EDCs in larval and fetal animals, as well as in young and adults.

2.3.1 Steroid Synthesis and Receptor Agonists and Antagonists

33. Natural (e.g., B, F) and synthetic GCs (e.g., dexamethasone, prednisone) have been reported in some wastewater effluents (Chang et al., 2007; Liu et al., 2011; Schriks et al., 2010; Van der Linden et al., 2008) and in surface waters (Chang et al., 2007; Liu et al., 2011; Van der Linden et al., 2008). 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 B in cultured rat adrenal cells (Ziolkowska et al., 2006), although a separate study reported inhibition of P450c21 following chronic exposure to resveratrol (Supornsilchai et al., 2005).

34. Glycyrrhetinic acid, the distinctive compound found in liquorice, stimulates production of adrenal DHEAS through induction of sulfotransferase 2A1 (Al-Dujaili et al., 2011).

35. Treatment of hypercortisolism (Cushing’s disease) may involve treatment with metyrapone, an inhibitor of P45011β, or ketoconazole, an anti-fungal drug used to treat athlete’s foot and similar problems that also blocks glucocorticoid synthesis (Feelders et al., 2010). Metyrapone treatment on an experimental basis also has proven useful in blocking emotional memories, such as those that occur with post-traumatic stress disorder (Marin et al., 2011). Metyrapone is effective in other vertebrates but is particularly toxic to salmonid fishes and should be considered a potential threat should it appear in wastewater effluents.

2.3.2 Metals

36. Long-term exposure to cadmium interferes with the ability of ACTH to stimulate interrenal tissue of rainbow trout, Oncorhynchus mykiss (Hontela, 1998; Norris and Hobbs, 2006) 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 (Norris et al., 1997; Norris et al., 1999; Norris, 2000). Other heavy metals may disrupt adrenal function in fish as well (Gagnon et al., 2006; Hontela, 1998). In addition to the adverse effects of cadmium on reproduction in mammals, cadmium also has direct inhibitory effects on B levels in rats (Jackl and Kollmer, 1982) and on guinea pig adrenal cell functions (Colby et al., 1987; Min et al., 2008).
2.3.3 Neuroactive Chemicals

37. The presence of a variety of neuroactive pharmaceuticals (e.g., fluoxetine, sertraline that can affect 5-hydroxytryptamine receptors and neuroendocrine (5-HT and NE) pathways) and their accumulation in wildlife (Schultz et al., 2010) offers more potential routes for interruption of the HPA axis. Fluoxetine reduces escape behavior at environmentally relevant levels in fathead minnows, *Pimephales promelas* (Painter et al., 2010) and reduces aggressive behavior in blue-head wrasse, *Thalassoma bifasciatum* (Semsar et al., 2004). Levels of B are reduced in rats exposed to fluoxetine (Elakovic et al., 2010).

2.3.4 Vasopressin Receptor Agonists and Antagonists

38. Drugs that mimic AVP (e.g., desmopressin and terlipressin) or antagonize AVP actions e.g., vaptans; Elthassan and Schrier, 2011) are potential EDCs. Several pollutants also interfere with AVP actions in peripheral mammalian systems, such as PCBs and PBDEs see Kodavanti and Curra-Collazo, 2010). 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

39. The CRHR₁ antagonists DMP696 and DMP904, developed for treatment of anxiety disorders (Li et al., 2005), may appear in wastewater. These chemicals could pose a threat to HPA axis of wildlife.

2.3.6 Pesticides

40. Endosulfan decreases the responsiveness of dispersed adrenocortical cells from rainbow trout to ACTH (Dorval et al., 2003). Derivatives of DDT (DDD and DDE, respectively) also reduce the responsiveness of adrenal cells of rainbow trout (Benguira et al., 2002; Leblond and Hontela, 1999) and tilapia, *Sarotherodon aureus* (Ilan and Yaron, 1980) to ACTH, as well as reduce the HPA axis response to stress in tilapia (Ilan and Yaron, 1983) and arctic char, *Salvelinus alpinus* (Jorgensen et al., 2001). Most pesticides, however, have not been tested for their ability to affect the HPA axis, but because of observed effects of E2 and testosterone on the HPA axis, one might expect a number of pesticides already shown to disrupt reproduction may also affect the HPA axis see Section 2.3.8, below).

2.3.7 Arylhydrocarbon Receptor (AhR) Agonists

41. PCBs, known activators of the AhR, reduce the responsiveness of arctic char, *Salvelinus alpinus* (Jorgensen et al., 2002); yellow perch, *Perca flavescens* (Hontela, 1998); and tilapia, *Oreochromis mossambicus* (Quabius et al., 1997) to stress. PCBs as well as PDBEs interfere with the actions of AVP on ion balance in mammals (Kodavanti and Curra-Collazo, 2010), 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 (Kodavanti and Curra-Collazo, 2010).

42. Acute treatment with β-naphthoflavone, another AhR agonist, decreases responsiveness of rainbow trout adrenal cells to ACTH (Wilson et al., 1998; Aluru and Vijayan, 2004), as well as the response of liver cells to cortisol (Aluru and Vijayan, 2004). However, more recent studies of acute BNF treatment of rainbow trout show activation of 5-HT turnover in the hypothalamus (Gesto et al., 2008) and elevation of plasma F and plasma glucose, as well as increased liver glycogenolysis and gluconeogenesis (Tintos et al., 2008). Acute BNF exposure also decreased the F response to handling stress (Wilson et al., 1998).
2.3.8 Estrogenic and Androgenic Disruptors on the HPA Axis

43. Compounds in bleached kraft mill effluent (BKME), recognized for its androgenic actions on female fish, cause atrophy of pituitary corticotropes and adrenocortical cells and reduce the normal response to stress in yellow perch (Hontela et al., 1997). Both E_2 and the weaker estrogenic nonylphenols reduce plasma cortisol levels in the gilthead bream, _Sparus auratus_ (Teles et al., 2005), and other estrogenic chemicals may also affect the HPA axis. Testosterone also influences hypothalamic synthesis of AVP and CRH (Viau et al., 1999).

2.4 In-vitro Screening Assays

44. Potential in-vitro assays can be separated into biochemical assays conducted with cellular extracts or specific molecules and studies utilizing intact cells or tissue slices. These procedures mostly are useful for EDCs that are GR agonists or antagonists in non-mammals.

2.4.1 Computerized Receptor Assay

45. A computerized system has been employed that compares the 3-dimensional (3D) shape of a chemical with its potential fit into specific hormone receptor sites has great promise for rapid screening of chemicals (Spreafico et al., 2009; Vedani et al., 2009). Some of the chemicals tested with that show considerable affinity for mammalian GRs include amlodipine (a calcium channel blocker), clopidogrel (anti-platelet agent) dimoxystrobin (pesticide), E100 and E104 (food dyes), methylbenzylidene camphor (UV filter), oseltamivir (anti-viral drug), and trimipramine (psychotropic drug). The authors have data available for 2000 chemicals that they have screened for AhR and steroid receptors binding. Although binding ability only provides a preliminary screening at one level in the HPA axis, this procedure appears to be a superior screening approach for estimating relative potency to bind to the GR receptor because it requires no use of animals. Of course, this approach does not tell us whether the chemical will activate or inactivate the receptor. Furthermore, chemicals that have allosteric binding sites on receptors would be missed. Hence, additional cellular or in vivo testing will be required.

2.4.2 Competitive-binding Receptor Assays

46. Although receptors for CRH, AVP, ACTH, and GCs have been isolated and well-characterized from mammals, there is little information available about non-mammalian receptors other than GRs. The relative affinity of chemicals for mammalian receptors may provide useful information for non-mammals since the mammalian HPA hormones are structurally very similar to those of non-mammals, and the mammalian hormones are effective when tested in non-mammals. Hence, the use of mammalian competitive-binding assays may provide a rapid, easily standardized, and relatively inexpensive preliminary screening technique for potential HPA EDCs. However, additional tests in non-mammals may be necessary.

2.4.2.1 Cell Culture Systems

47. Examination of dispersed or cultured cells from any level of the HPA axis can provide better information than simple biochemical assays because an intact cell is much more complicated than an isolated reaction and, hence, once step closer to examining the intact organism.

Corticotropes

48. Corticotropes isolated from mammals (Adatia et al., 2002; Myers and Myers, 2000; Xie et al., 1999) 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).

**Adrenal Cortical Cells**

49. 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 (Hanley and Arlt, 2006). A human adenocarcinoma cell line (H295R) has been developed as a screening assay for chemical factors that interfere with steroidogenesis (Hecker et al., 2006) using production of progesterone, testosterone, and E2 as end products, and this system possibly could be validated for measuring GCs as well. An in-vitro method for assessing pesticide effects on adrenal cells of rainbow trout has been reported recently (Hontela et al., 2008) and could provide the basis for a simple screening assay for specifically directed at fish.

**GC Target Cells**

50. Some in-vitro systems have been described for looking at metabolic actions of GCs on mammalian uterine cells (Davies et al., 2006) liver cells (Bailly et al., 2009; Nader et al., 2010; Nguyen et al., 2010) (Yabualuri & Bashyam 2010; Visser et al., 2010) or adipose cells (Campbell et al., 2011; Drake et al., 2010). Additional in vitro systems have been explored in fish liver (Pierce et al., 2010; Smith and Wilson, 2010).

2.4.3 Gene-activation Assays

51. Expression of genes following exposure of a cell to a GC, GC antagonist, or GC agonist can be monitored by isolating mRNA, forming the corresponding cDNA, and identifying the specific genes involved using DNA microarrays. DNA technology has resulted in the development of DNA microarrays for various species for whom the genome has been cataloged (e.g., human, Xenopus, zebrafish). These microarrays are available from commercial sources, although they are relatively expensive. However, many of the genes activated in these arrays have yet to be linked to a known GC function. Chemicals can then be added to cells in the presence or absence of a GR agonist or antagonist to see if the tested chemical mimics, enhances, or antagonizes expression of specific genes or combinations of genes involved in the normal action of the GC.

2.4.4 In-vivo Screening Assays

52. In-vivo assays may be conducted using a variety of vertebrates, but typically, only intact fish, rats, or mice, and occasionally amphibians are employed. Because these assays would require incredible numbers of vertebrate animals to test the vast array of chemicals present in the environment, it would seem that in-vivo assays should be reserved only for definitive testing of chemicals that proved positive in the in-vitro Tier 1 screening assays.

53. 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 (Norris, 2000). For example, F 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 (Norris et al., 1997). 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 (Norris
et al., 1999). Furthermore, the exposed fish were unable to maintain elevation of ACTH and F beyond 12 hr during the 24-hr stress test whereas the unexposed fish could. Measurement of F levels in stressed and unstressed fishes 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 simply way to obtain meaningful information without the more laborious measurements of metabolic or immunological assessments. However, this approach might not detect effects on GC target cells. Hence, coupling this approach with a metabolic measurement such as plasma glucose may be desirable.

2.4.4.1 Fish

54. Most in vivo work has been done to assess HPA axis functions in trout or other larger species of fishes. Small species models such as fathead minnows or zebra fishes would be more efficient than using larger species as proposed by Scholz and Mayer (2008), but it would be more difficult to acquire sufficient volumes of plasma to undertake both hormone assays. Histological procedures might be employed at the hypothalamus, pituitary, or adrenal tissue, but this would be time consuming. Use of larger species would allow for examination of more HPA-related parameters than could be done with smaller species.

2.4.4.2 Amphibians

55. Since the HPA axis and the HPT axis are responsible for controlling metamorphosis in amphibians, the Tier 1 in-vivo assay employing Xenopus laevis tadpoles (e.g., Sugiyama et al., 2005) could be utilized for HPA EDC screening. However, one could have difficulty distinguishing between the effects of endosulfan operating via the HPA axis (Goulet and Hontela, 2003) from endosulfan affecting the HPT axis, as recently reported for endosulfan exposure (Brunelli et al., 2009).

2.4.5 Challenges and Limitations

56. The emphasis of endocrine-disruption studies primarily has been on the hypothalamo-pituitary-gonadal (HPG) axis and secondarily on the hypothalamo-pituitary-thyroidal (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 Tier 1 protocols for screening potential HPA disruptors.

2.4.5.1 In-vitro vs In-vivo Assays

57. In-vitro (ex-vivo) systems have a number of advantages for identification of disruptors of the HPA axis. They are relatively simple, highly selective, and very sensitive systems that can be easily controlled and standardized for use in different laboratories. In addition, commercial kits often can be made that create further standardization and ease of operation. However, whole organisms are much more complex than biochemical or cellular models that do not monitor interactions of effects at numerous levels, including the brain, the pituitary, the endocrine target gland, and the peripheral targets of these hormones. Variables such as metabolism and/or excretion rates, plasma-binding proteins, target tissue receptor levels, and feedback effects are present in vivo but not in vitro.

58. Furthermore, the factors that make in-vitro assays simple tools are also their major weakness. In-vivo observations often differ markedly from predictions based on in-vitro assessments. For example, bisphenol A binds rather poorly to the estrogen receptor (ER) when compared to E\textsubscript{2} binding in vitro, but has proven to be an important endocrine disruptor in vivo (Diamanti-Kandarakis et al., 2009). Similarly, ethinylestradiol EE\textsubscript{2} found in most birth control pills is equipotent to E\textsubscript{2} with respect to binding to the mammalian ER, yet EE\textsubscript{2} is 10 times more potent than E\textsubscript{2} in fish (Vajda and Norris, 2011). Hence, in vitro
systems may be useful for quick preliminary screenings of possible disrupting activities, but weak activity should not be considered proof of safety until a chemical is tested in the appropriate species \textit{in vivo}.

\textbf{2.4.5.2 Mixtures vs. Single Chemicals}

59. Most laboratory studies emphasize examination of the effects of a single chemical at various doses on one aspect of the endocrine system. Screening programs for EDC activity take a similar approach. However, in nature, animals are exposed to mixtures of chemicals that may produce additive or antagonistic effects operating through the same or different cellular mechanisms at different levels of organization within the HPA axis (Diamanti-Kandarakis, 2009; Hamlin and Guillette, 2011). Hence, every wastewater discharge represents a unique and dynamic collection of chemicals that are presented to wildlife. Although the focus of this discussion is the monitoring of chemicals for potential identification of EDCs, it is important to keep in mind the limitations of screening chemicals one at a time in lieu of the chemicals to which animals are exposed.

\textbf{2.4.5.3 Other Parameters to Be Considered}

60. 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 fishes to Cd may increase cortisol release, whereas longer treatments decrease the sensitivity of adrenal cells to ACTH (Hontela, 1998). Similarly, short-term (Ziolkowska et al., 2006) and long-term (Supornsilchai et al., 2011) 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 (Hontela, 1998; Norris, 2000). However, stressing of fish may yield opposite effects to exposure observed for unstressed fish or for fed vs unfed fish (Jorgensen et al., 2002).

61. Many vertebrates exhibit marked seasonal variations in HPA activity (Romero, 2002). Typically, peak levels of GCs 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.

62. Finally, investigators must recognize the complexity of the HPA axis when devising testing protocols, especially \textit{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 Tier 1 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, indicates that in-vitro assays can never be considered definitive for screening of chemicals for potential HPA axis activity.
3. The Somatotropic Axis

3.1 Overview

63. The somatotropic axis is responsible for the release of growth hormone and insulin-like growth factor. These hormones regulate a variety of functions related largely 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 3-1). 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.

64. 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 3-1). In rodents and humans, growth hormone secretion occurs in a pulsatile fashion (Eden, 1979; Veldhuis et al., 2000). 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 at the brain by a neonatal pulse in testosterone production (Gustafsson and Stenberg, 1974).

65. Growth hormone is delivered to peripheral tissues, where it binds to cell surface receptors that initiate a phosphorylation cascade that involves the JAK/STAT pathway (Carter-Su et al., 1996). Elevated growth hormone levels result in insulin resistance, increased blood glucose, increased lipid metabolism (Perrini et al., 2008). Tissue responses to growth hormone are dependent upon both the amount of circulating hormone and its pattern of production. 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 factors 1 and 2 (IGF-1, IGF-2). IGF-1 is the primary cell-signaling form of IGF (Figure 3-1).

66. IGF-1 is largely responsible for the growth-promoting activities associated with the somatotropic axis, exerting multiple effects at various tissues relating to growth (Giustina et al., 2008; Goldspink, 2007; Wood et al., 2005). In fish, amphibians, and mammals, IGF-1 and/or IGF-2 contribute to spermatogenesis and/or oocytes maturation (Kagawa et al., 1994; Nader et al., 1999; Sadler et al., 2010). Both IGF-1 and IGF-2 also appear to contribute to fetal development in mammals (Gluckman and Pinal, 2003). Serum IGF-1 levels positively correlate to birth weight (Gluckman and Pinal, 2003) and fetal IGF-1 deficiency results in low birth length. IGF-1 also contributes to osmoregulation in fish (McCormic, 2001) and to reproductive performance in cattle (Velazquez et al., 2008). A summary of physiological responses to suppression or excitation of the somatotropic axis is presented in Table 3-1.
Figure 3-1. The Somatotropic Axis

Table 3-1. Some Physiological Consequences of Aberrant Suppression and Activation of the Somatotropic Axis (Summarized from Melmed and Kleinberg [2002] for Mammals Unless Indicated Otherwise)

<table>
<thead>
<tr>
<th>Suppression</th>
<th>Excitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased body fat</td>
<td>Increased body size/stature in fish (Eales et al., 2004)</td>
</tr>
<tr>
<td>Abnormal lipid profile</td>
<td>Heart disease</td>
</tr>
<tr>
<td>Impaired cardiac function</td>
<td>Thyroid dysfunction in fish (Eales et al., 2004)</td>
</tr>
<tr>
<td>Reduced muscle mass</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Menstrual disturbances</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>Sea water tolerance in fish (McCormic, 2001)</td>
</tr>
<tr>
<td>Immunodeficiency</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Consequences of Disruption by Environmental Chemicals

67. Physiological responses to suppression or excitation of the somatotropic axis are known primarily from disease conditions in humans and from laboratory studies involving ablation of the pituitary gland or administration of exogenous hormone (IGF-1). 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 (Du et al., 1992; Kopchick and Cilffi, 1991). However, disruption of the somatotropic axis in response to environmental chemicals has received relatively little attention, despite its multi-faceted role in physiology.

3.2.1 Estrogenic Chemicals

68. 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 (Carnevali et al., 2005; Davis et al., 2007; Filby et al., 2006). 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 (Jiao and Cheng, 2010; Krattenmacher et al., 1994; Shved et al., 2008). This regulatory influence of estrogens in the somatotropic axis has been demonstrated in both mammals and fish. Reduced growth and disrupted smoltification are effects associated with the exposure of fish to estrogenic chemicals (Servos, 1999) and may be the consequence of the negative regulation of the somatotropic axis by estrogens.

3.2.2 Anti-thyroid Chemicals

69. Thyroid hormone increases somatotropic axis signaling in mammals, birds, and fish (Ikeda et al., 1991; Schmid et al., 2003; Tsukada et al., 1998). Thyroid hormone may stimulate the somatotropic axis through its induction of pituitary growth hormone synthesis (Koenig et al., 1987) or through direct action on hepatic IGF-1 synthesis (Leung et al., 2008). 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, bisphenol A has been shown to bind the thyroid hormone receptor in an antagonistic manner, thus preventing thyroid hormone signaling (Moriyama et al., 2002). 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 (Aluru et al., 2010). Similarly, anti-thyroidal polychlorinated biphenyls (PCBs) (Collins et al., 1977; Iwasaki et al., 2002) reduced expression of IGF-2 levels in the liver of adult mink (Mustela vison) (Backlin et al., 1998). PCB exposure has been shown to have adverse effects on parameters of growth, including bone development (Mauck et al., 1978).

3.2.3 Corticosteroid Stimulants

70. Corticosteroids suppress somatotropic axis signaling in fish and mammals (Kajimura et al., 2003; McCarthy et al., 1990). 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). As such, exposure to these chemicals would likely suppress IGF-1 levels. Increased plasma corticosteroid levels may sometime 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 (Fraites et al., 2009). 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 (Waring and Moore, 2004). This effect is consistent with the action of IGF-1 on osmoregulation in fishes.

### 3.3 Chemicals that Directly Disrupt the Somatotropic Axis

71. We are aware of no non-peptide chemicals that interfere, as agonist or antagonists, with growth hormone or IGF interactions with their respective receptors. However, 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 (Reinecke, 2010). Monitoring of the somatotropic axis may have value in controlled laboratory experiments but may have limited use in field applications.

### 3.4 Assays

#### 3.4.1 In-vitro Assays

72. *In vitro* assays described elsewhere in this document for evaluating interactions of chemicals with estrogen, thyroid, and glucocorticoid signaling would be informative of possible effects on the somatotropic axis as well.

73. Le Gac et al. (2001) 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 is 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.

74. Elango et al. (2006) 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 17\(\beta\)-estradiol or o,p\(^{\prime}\)-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.

#### 3.4.2 In-vivo Assays

75. Analysis of plasma levels or hepatic expression of IGF-1 during *in vivo* assays would be informative of endocrine disruption via action on the somatotropic axis (Aluru et al., 2010; Carnevali et al., 2005; Davis et al., 2007; Filby et al., 2006; Kajimura et al., 2003). Hepatic IGF-1 mRNA are typically measured by qPCR, whereas plasma IGF-1 levels are measured by radioimmunoassay. Analysis of growth hormone levels would be less informative due to the pulsatile nature of growth hormone secretion (Melamed et al., 1995; Robinson and Hindmarsh, 2011).

### 3.5 Strengths, Challenges, and Limitations

76. 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 3-1). 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 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 somatotropic axis. This disruption could then be confirmed in in vivo screening assays (e.g., Level 4 assay of the Conceptual Framework) or life-cycle studies (e.g., Level 5 assays of the Conceptual Framework) by evaluating IGF-1 levels in the test organisms as described above. Alternatively, IGF-1 levels could be measured in screening assays (e.g., Level 3 assays of the Conceptual Framework) as a first indication that a chemical may have endocrine-disrupting properties since multiple endocrine targets converge on this endpoint. 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.
4. The Retinoid Signaling Pathway

4.1 Overview

77. 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 and the RXR in vertebrates. Excess or suboptimal levels of retinoids during development results in developmental abnormalities (Ross et al., 2000).

4.1.1 Retinoic Acid Receptor (RAR) Signaling

78. The RAR (NR1B1) is found in vertebrates and chordates, but thus far, has not been identified in protostome invertebrates (Marletaz et al., 2006). 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).

79. Excessive RAR-mediated signaling, attained through prenatal, perinatal, and postnatal exposure to exogenous retinoid results in a variety of development abnormalities (Collins and Mao, 1999; Elmazar et al., 1996; Elmazar et al., 2001). These include brachial arch and neural tube defects in mammals (Lee et al., 1995; Menegola et al., 2004); limb malformations in frogs (Degitz et al., 2000); and fin deformities in fish (Miwa and Yamano, 1999; Vandersea et al., 1998). Reduced RAR signaling has been shown to cause abnormalities in diaphragm development in rats (Clugston et al., 2010), blood vessel and bone development in fish (Hayashida et al., 2004), and impaired lens regeneration in frogs (Ptonis et al., 2000).

4.1.2 The Retinoid X Receptor Signaling Network

80. The RXR NR2B) is an ancient member of the nuclear receptor family and is expressed in lineages ranging from jellyfish (cnidarians) to humans (Kostrouch et al., 1998). RXR functions as a master switch in coordinating the activities of multiple components of signaling pathways involved in processes, including development (Yu et al., 1991), reproduction (Wendling et al., 1999), lipid homeostasis (Chawla et al., 2001), and metabolism. RXR can self-dimerize forming a homodimeric complex that is activated by ligands such as 9-cis retinoic acid docosahexaenoic acid DHA). RXR also can form heterodimeric complexes with a variety of nuclear receptors (Figure 4-1). Vertebrate RXR heterodimers have been categorized as permissive or nonpermissive (Shulman and Mangelsdorf, 2005). 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 (Mu et al., 2000; Li et al., 2002). Example permissive partners to RXR include the PPAR, the liver X receptor (LXR), and the farnesoate X receptor (FXR) (Shulman and Mangelsdorf, 2005). 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)) (Shulman and Mangelsdorf, 2005). However, activation of RXR by its ligand can result in the synergistic activation of the liganded partner. Noteworthy in this respect is the observed synergistic activation of retinoid signaling when both RXR and RAR are ligand-bound by agonists (Elmazar et al., 2001; Minucci et al., 1997).
81. 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.

4.1.2.1 Reproduction

82. 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 (Huang, 2008). 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 transduces the retinoic acid signal that determines whether a gamete will develop into a spermatogonium or an oocyte (Bowles et al., 2006). RXR null mice are infertile (Kastner et al., 1996). Inappropriate post-partum signaling of RXR:PPAR in the mammary gland results in the production of toxic milk resulting in neonate death (Wan et al., 2007; Yang et al., 2006).

4.1.2.2 Development

83. 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 (Sucov et al., 1994). Mice containing an RXRa 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 (Chen et al., 2009). Similar adverse effects on brain development occur with thyroid hormone deficiency (del Escobar et al., 2004). 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 (Forrest et al., 2002). Clearly, the entire vitamin/hormone signaling network involved in brain development requires further elucidation. The RXR:EcR heterodimer coordinates multiple developmental processes in arthropods (e.g., insects, crustaceans). These include vitellogenin production for provision to embryos (Hannas et al., 2010), embryo development (Mu and LeBlanc, 2002), growth, and metamorphosis (LeBlanc et al., 1999).

4.1.2.3 Lipid Homeostasis

84. 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) (Chawla et al., 2001). These receptors typically regulate genes that govern uptake, synthesis, transport,
storage, metabolism, and elimination of specific lipid classes (Chawla et al., 2001; Shulman and Mangelsdorf, 2005). Disruption of the RXR node within this network is associated with metabolic syndrome and associated disorders (Shulman and Mangelsdorf, 2005). 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 (Rees et al., 2008).

4.2 Disruption of Retinoid Signaling by Xenobiotics

85. 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.

4.2.1 Reductions in Retinoid Levels

86. 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 (Kelley et al., 1998), presumably resulting from the mobilization of retinoids from retinyl ester stores (Brouwer et al., 1989; Jurek et al., 1990), ultimately resulting in the increased renal excretion of polar retinoid derivatives (Brouwer et al., 1989; Hakansson and Ahlborg, 1985).

4.2.2 RAR Agonists

87. RAR agonist, 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 (Kamata et al., 2008; Nishikawa et al., 2003). RAR also was activated by the pesticides aldrin, chlordane, dieldrin, endrin, and endosulfan in a transactivation assay (Lemaire et al., 2005). 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 (Kamata et al., 2008).

4.2.3 RXR Agonists/Antagonists

88. RXR also has been shown to be activated in a two-hybrid assay by various xenobiotics (Li et al., 2008). Among the more potent agonists were 2-t-butylphenol, tetrabromobisphenol, 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 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.

89. The most potent RXR agonist identified to date is tributyltin. This compound has been shown to activate RXR in transactivation assays at nanomolar concentrations (Bouton et al., 2005; Grun and Blumberg, 2006). 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 (Cui et al., 2011; Grun et al., 2006). In contrast to activation of these permissive receptor complexes, non-permissive receptors (e.g., RAR, TR, VDR, PXR) are not activated by tributyltin (Grunet al., 2006). 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 organotinns typically have no (butyltin) or lesser (dibutyltin, tetrabutyltin) activity (Grun et al., 2006). RXRs derived from various species, including mammals, amphibian, and even invertebrates are activated by tributyltin (Grun et al., 2006; Kanayama et al., 2005; Nishikawa et al., 2004; Wang and LeBlanc, 2009). The high potency with which tributyltin activates the RXR stems from its forming covalent bonds within the receptor ligand-binding domain (le Maire et al., 2009).

### 4.3 Consequences of Disrupted Retinoid Signaling

90. There exists many reports of associations among environmental pollutant, alter retinoid levels in exposed wildlife, and physiological responses consistent with altered retinoid signaling. Fish white sucker, *Catostomus commersonii* collected from a polluted site had reduced hepatic retinol and retinyl palmitate levels as compared to fish sampled from a reference site (Branchaud et al., 1995). Reduced retinoid stores was 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 (Besselink et al., 1998). Common terns (*Sterna hirundo*), feeding on fish from polluted areas produced offspring with decreased retinol ester levels and elevated EROD activity (Murk et al., 1996). Affected chicks experienced longer incubation times and reduced body weight at hatching. These examples are highly indicative of exposure to Ah receptor agonists.

91. 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 (Grun et al., 2006; Kirchner et al., 2010). 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 (Kirchner et al., 2010), indicating 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 (Grun et al., 2006). Tributyltin also stimulated increases in the expression of Fatp, Pck1, Acac, and Fasn in liver (Grun et al., 2006). 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 (Grun et al., 2006). Similar effects of tributyltin were observed in chronically exposed amphibians (*Xenopus laevis*) and fish (*Oncorhynchus tsawytscha*). Exposed frog tadpoles (1–10 nM aqueous exposure) experienced a dose-dependent increase in ectopic adipocyte formation (Grun et al., 2006); while exposed Chinook salmon experienced increased body mass, plasma triacylglycerols, cholesterol, and lipase activity, with increasing tributyltin dose (Meador et al., 2011). 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.

### 4.4 Assays

#### 4.4.1 Reporter Assays for the Assessment of Ah Receptor Agonists

92. Ah Receptor 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 Ah receptor. Early versions of these assays involved measurement of the activity of enzymes induced by the Ah receptor in cultured cells following treatment with the chemical or in liver microsomes from rodents administered the chemical (Peturulis et al., 2001). Typically, the activity associated with the enzyme CYP1A1 was measures, such as ethoxycoumarin O-deethylase activity. More recently transcription reporter
assays have been constructed and used to detect Ah receptor 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 (Olsman et al., 2007).

93. 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 Ah receptor 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 measure 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 Ah receptor. Many reporters are currently available from commercial sources (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, lost cost, amenability to high-throughput applications, and rapid assay time. Example Ah receptor reporter assays are described in Table 4-1.

4.4.2 Alterations in Retinoid Levels and Metabolism In Vivo

94. Endogenous retinoid levels can be severely depleted by Ah receptor 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 [TG 440], Hershberger assay [TG 441], two-generation toxicity assay [TG 416]); fish (fish reproductive screening assay [TG229], fish screening assay [TG230]; androgenized female stickleback screen, Medaka multigeneration test) (Ndayibagira et al., 1995; Palace et al., 2001); amphibian assays (Xenopus embryo thyroid signaling assay, amphibian metamorphosis assay (TG 231) (Gutleb et al., 1999; Leiva-Presa et al., 2006); and avian assays (Avian 2 generation reproductive toxicity assay) (Boily et al., 1994; Palace et al., 2001).

95. 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 (Schmidt et al., 2003). Typically, exposure to Ah receptor agonists decrease retinoid and retinoid ester levels in the liver and increase levels in the kidney (Fletcher et al., 2005), though variability can exist between species and strain (Nilsson and Hakansson, 2002). A promising biomarker of retinoid disruption by Ah receptor ligands is the loss of the retinol metabolite 9-cis-4-oxo-13,14-dihydroretinoic (DHRA) acid in liver tissue (SchmidtHoegberg et al., 2003). 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 (Fletcher et al., 2005; Schmidt et al., 2003). However, the occurrence and behavior of this metabolite in non-roden species is presently not known.

4.4.3 RAR Agonists/Antagonists (In-vitro Reporter Assays)

96. Reporter assays have been used for two decades to evaluate retinoid-like activity of chemicals (Bernard et al., 1992). Early reporter assays utilized chloramphenicol acetyltransferase (CAT) as the reporter gene; however, more contemporary assays use reporter genes that code for fluorescent proteins (Moise et al., 2009). RAR reporter assays are commercially available (Invitrogen, Qiagen Company). Commercial screening services using RAR reporter assays are also available (INDIGO Biosciences). Binding assays have also been used with expressed RAR proteins to assess interactions between receptor and putative ligands (Bernard et al., 1992; Lemaire et al., 2005). 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.
4.4.4 RXR Agonist/Antagonists (In-vitro Reporter Assays)

Transcription reporter assays have been used to assess both agonistic and antagonistic activity of putative RXR ligands (Goldstein et al., 2003; Li et al., 2008; Schoff and Ankley, 2004). 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 (Nishikawa et al., 2004), the insecticide metabolite methoprene acid (Harmon et al., 1995; Schoff and Ankley, 2004) and (unidentified) metabolite(s) of BPA (Li et al., 2008) also activate RXR, but with much lower affinity.

Table 4-1. Example Transcription Reporter Assays that Are Used to Evaluate Activation of the Ah Receptor by Chemicals

<table>
<thead>
<tr>
<th>Species</th>
<th>Cells</th>
<th>Reporter Gene</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>HepG2 hepatoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Human</td>
<td>MCF7 breast tumor</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Human</td>
<td>LS180 intestinal epithelial</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Human</td>
<td>HepG2 hepatoma</td>
<td>Firefly luciferase pLuc1A1</td>
<td>(Postlind et al., 1993)</td>
</tr>
<tr>
<td>Rat</td>
<td>H411e hepatoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>GPC16 intestinal adenocarcinoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Hamster</td>
<td>AHL lung</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Mouse</td>
<td>H1L.1.1c2 hepatoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Mouse</td>
<td>MLEL1.1c1 hepatoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Garrison et al., 1996)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Hepa 1c1c7 hepatoma</td>
<td>Green fluorescent protein pGudLuc1.1</td>
<td>(Nagy et al., 2002)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>RTH-149 hepatoma</td>
<td>Firefly luciferase pGudLuc1.1</td>
<td>(Richter et al., 1997)</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>COS-1 monkey kidney*</td>
<td>Firefly luciferase pGL4-ckCYP1A5-6XRE</td>
<td>(Karchner et al., 2005)</td>
</tr>
</tbody>
</table>
| *Cells are transfected with the zebrafish AhR and ARNT expression constructs.

4.4.5 Adipocyte Differentiation Assay

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 (Grun et al., 2006; Kanayama et al., 2005). 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 (Grun et al., 2006). Oil red O stains lipids that accumulate in the adipocytes. In addition, triglyceride levels can be measured in cells using commercially available assays (Kanayama et al., 2005). Markers of adipocyte differentiation, such as induction of
PPARγ and AP2 mRNA levels (Lehmann et al., 1995) 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.

### 4.4.6 Microarrays

99. 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 antagonist. Microarrays have been used extensively to evaluate changes in gene expression among cells exposed to RXR agonists (Gupta et al., 2001; Li et al., 2003; Liu et al., 2000; Rusiniak et al., 2000; Soref et al., 2001; Tamayo et al., 1999; Ueda, 2001). However, significant variability in gene responses have been noted, and these differences attributed to cell type used, agonist used, arbitrary selection of threshold response levels, and lack of intra-experiment replication (van der Spek et al., 2003). Analyses of gene expression networks through the use of microarrays holds promise as a holistic tool to assess endocrine disruption via RXR and other pathways. However, standardization of methods are required before the approach can be adopted for routine use.

### 4.4.7 Alterations in Lipid Levels and Metabolism (In vivo)

100. Changes in lipid levels among mammals used in existing OECD assays and perhaps 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 (Repa et al., 2000). Cholesterol absorption can be measured by providing radio-labeled cholesterol to the test animals and measuring radioactivity in feces as well as in serum (Schwarz et al., 1998), while ABC1 transporter levels can be measure using standard immunoblotting or RT-PCR techniques (Repa et al., 2000).

### 4.5 Strengths, Challenges, and Limitations

101. Considering its obligatory role in several endocrine-signaling processes due to its obligate heterodimerization with other nuclear receptors (see Figure 4-1), RXR signaling should have a prominent position 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 Ah receptor agonist/antagonists, which have the potential to modify retinoid hormone levels (see Table 4-1) and RAR agonists/antagonists, which have the potential to disrupt various developmental processes.

102. 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.

103. Adipocyte differentiation assays hold promise as a screening tool, both in culture cells and in the whole organism. However, endpoints related to adipocyte differentiation may prove to be more holistic markers of disruptions in lipid homeostasis that may be due to a variety of endocrine and non-endocrine processes.
5. The Thyroid-Releasing Hormone:Thyroid Stimulating Hormone:Thyroid Hormone Signaling Pathway

5.1 Introduction

104. Thyroid hormones are essential for normal physiological functions, including neurodevelopment, growth, and cellular metabolism. Over the course of the last 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 tyrotropin 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 disruptors (TDs). Considering the critical role of thyroid hormones in key physiological processes, it is important to accurately tests for potential thyroid toxicants. In 2007, Zoeller et al. (2007) 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. (2007).

5.1.1 Brief Review of the Hypothalamus:Pituitary:Thyroid (HPT Axis)

105. Thyroid endocrinology is well conserved across vertebrate taxa. This includes aspects of thyroid hormone synthesis, metabolism, and mechanisms of action (Zoeller et al., 2007). 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 paraventricular nucleus (PVN) are the primary site of TRH production (Jackson et al., 1985; Lechan et al., 1986). 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 (Chiamolera and Wondisford, 2009). 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 (Fliers et al., 2006). However, in teleosts, the external zone of the median eminence directly innervates the pars distalis of the pituitary (Galas et al., 2009). 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) (Galas et al., 2009). Interestingly, in some teleost species and amphibians, TRH does not affect thyroid stimulating hormone (TSH, thyrotropin) secretion. Rather, corticotropin releasing hormone acts as a TSH releasing factor (Larsen et al., 1998)

106. 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 (Grossmann et al., 1997; Shupnik et al., 1989). The α subunit is common to TSH, follicle stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (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, although paracrine and autocrine activity has been recently described for TRH secreted in the anterior pituitary (Bruhn et al., 1998). 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 (Gehret et al., 2010). Activation of TRHR by THR 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 (Cohen et al., 1999; Steinfelder et al., 1992; Zanger et al., 1999). In addition, TRH stimulates post-translational glycosylation of TSH, which is critical for TSH heterodimerization, secretion, and bioactivity of mature TSH (Haisenleder et al., 1992; Harel et al., 1993).

107. TSH released from the anterior pituitary binds to receptors on the cell surface of thyroid follicle cells (Manley et al., 1982). 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 cAMP-responsive element binding (CREB) (Corda et al., 1989). There is some evidence that TSH additionally activates protein kinase C (PKC) and diacylglycerol (DAG) signaling pathways (Rivas and Santisteban, 2003). The effects of receptor activation are multifunctional, including increased uptake of iodide into the thyroid cells, iodination of tyrosyl residues on thyroglobulin (TG), synthesis and oxidation of TG, TG uptake from thyroid colloid, and production of thyroid hormones T4 and T3 (Zoeller et al., 2007).

108. Iodine uptake in the thyroid gland is governed through the actions of the sodium-iodide symporter (NIS) (Carrasco, 2003; Dohan et al., 2003). 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-five times greater to 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 (Bizhanova and Kopp, 2009). NIS is also auto-regulated, where excess iodine accumulation suppresses NIS gene expression (Pisarev and Gartner, 2000). Once iodine molecules are transported into the cell, they are bound to tyrosine residues of thyroglobulin (TG) protein as either mono-iodothyronine (MIT) or di-iodothyronine (DIT). As with NIS, TG is under regulatory control of TTF1, TTF2, and Pax8 within the thyrocyte and, thus, de novo synthesis of TG production is stimulated by TSH (Damante and Di Lauro, 1994; Fabbro et al., 1994; Kambe et al., 1996). 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 TG (Kambe et al., 1996). Hydrogen peroxidase is produce through the activity of DUOX/ThOX oxidase enzymes located at the apical pole of the thyroid follicular cells (Pachucki et al., 2004; Wang et al., 2005). Thyroperoxidase (TPO) facilitates covalent attachment of iodide by reducing H\(_2\)O\(_2\) and oxidizing iodine where they bind to distinct tyrosyl residues on the thyroglobulin protein forming DIT or MIT (Pachucki et al., 2004; Wang et al., 2005). Two DIT molecules form T4 and one DIT and MIT molecule form T3.

109. 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 (Zoeller et al., 2007). Once in the blood stream, thyroid hormones are either bound to transport proteins, thyroid binding globulin (TGB), transthyretin (TTR), 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 TGB, 15% to TTR, and <5% to albumin (Refetoff et al., 1970). 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 (Mendel et al., 1987). 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, are responsible for local synthesis of T4 and T3 within the thyroid, peripheral and local conversion of T4 to T3 (the biologically active form of TH), breakdown of reduced T3 (rT3), and inactivation of T3 (Ng et al., 2009; St Germain et al., 2009). In addition to deiodination, thyroid hormones are metabolized in the liver and kidney through conjugation with sulfate or glucuronic acid (Ng et al., 2009; St Germain et al., 2009).

At the site of action, bioactive T3 either diffuses passively across the cellular membrane or is actively transported. 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 stereo-selective T4 and T3 transporters have been identified, including organic ion transport proteins (OATP) and members of the monocarboxylate transporter (MCT) family (Braun et al., 2010; Braun et al., 2011; Suzuki and Abe, 2008)). 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 (Braun et al., 2010; Oppenheimer et al., 1976). T3 binds to each of the TRs with near equal affinity and exhibits an approximately 50-fold greater affinity for TRs than T4 (Oppenheimer et al., 1994). However, there is some evidence of selective functional activation of T3 with each receptor that may be co-regulator dependent (Zoeller et al., 2007). 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 (Kouidhi et al., 2010; Laflamme et al., 2002). Conversely, in peripheral tissues, TH results in TR-ligand dependent activation of genes associates with development, growth, and metabolic control (Zoeller et al., 2007).

5.2 Mechanisms and Potential Points of Action for HPT-disrupting Compounds

Exposure to wide range of structurally diverse environmental chemicals, including PCBs; dioxins (tetrachlorodibenzo-p-dioxin, TCDD); and polychlorinated dibenzofurans (PCDFs); bisphenol A (4,4’ isopropylidenediphenol or BPA); triclosan; polybrominated diphenyl ethers (commonly known as flame retardants); phthalates, such as di[2-ethylhexyl] phthalate (DEHP), di-n-octyl phthalate (DnOP), diisodecyl phthalate (DIDP), di-n-hexyl phthalate (DNHP), and di-n-butyl phthalate (DBP); perchlorate; halogenated pesticides; and others such as parabens are known to disrupt thyroid axis signaling, homeostasis, and function (Grun et al., 2006; Jugan et al., 2010; Kanayama et al., 2005) (Grun et al., 2006). 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 (Patrick, 2009). Epidemiological studies support correlations of 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 in of circulating serum T4 and TSH levels (Patrick, 2009). Thus, modifications within the HPT axis have focused on molecular/physiological events that result in altered hormone levels. However, while TSH levels are an excepted 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 agonists/antagonists, which may have direct pleotropic effects.

Figure 5-1. Thyroid Axis and Known Sites of Action for TDCs

112. The thyroid system is a highly complex, and thyroid hormone homeostasis involves a complex network of homeostatic regulatory interactions (Kanayama et al., 2005). Thyroid-disrupting compounds 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 (Jugan et al., 2010; Kanayama et al., 2005). 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.

113. As illustrated in Figure 5-1, thyroid-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 thyroperoxidases T4/T3, modification in

Figure modified from Jugan et al., 2010. 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, glucuronosyltransferase.
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 TH and RXR receptors as direct agonists or antagonists.

114. At the top of the HPT axis, TSH signaling is adversely affected by TDCs. Using Chinese hamster ovary cells (CHO) transfected with the recombinant human thyrotropin (TSH) receptor, Santini et al., 2003 demonstrated that 1,1-bis-(4-chlorphenyl)-2,2,2-trichloroethan (DDT), Aroclor 1254 and lemon balm each inhibited TSH-stimulated cAMP production \textit{in vitro} (Santini et al., 2003). 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., 2009 demonstrated that DDT exerts an inhibitory effect through modification of TSHr intracellular trafficking, which is necessary for TSH signal transduction (Picchietti et al., 2009). 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 (Patrick, 2009). 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.

115. The effect of TDCs on the NIS receptor protein has been illustrated with several environmental chemicals, including, perchlorate, thiocyanate, bromate, and nitrate (De Groef et al., 2006). Each of these compounds compete with iodine for binding to the NIS transport protein inhibiting the uptake of iodine into the follicular thyroid cell (Wolff, 1998). PCBs, on the other hand, down regulate expression of NIS (Pocar et al., 2006). 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), ethylenethio- urea (fungicide), soy isoflavones, and benzophenone 2 inhibit the formation and/or activity of TPO. Inhibition of TPO impedes the ability of the follicular cell to synthesize thyroglobulin, thus decreasing synthesis of T4 and T3 (Kanayama et al., 2005).

116. TDCs may also impact circulating levels of free and bound thyroid hormones through their ability to bind with thyroid hormone transport proteins. PCBs, flame retardants, phthalates, and pentachlorophenol each bind to TTR. In their bound form, these chemicals compete with thyroid 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 (Boas et al., 2006). PCBs, flame retardants, bisphenol A, and dioxins modulate active transport and cellular uptake of thyroid hormones through disruption of hormone transport proteins, including MCT8 and OATP (Kanayama et al., 2005; Patrick, 2009). Richardson et al., 2008 found that PBDEs directly modify mRNA expression of monocarboxylate transporter 8 (Mct8) (Richardson et al., 2008). These and other studies suggest that exposure to TDCs may alter mechanisms associated with hormone uptake and biliary excretion. A likely mechanism for this is cross talk with other nuclear hormone receptors that regulate expression of phase one, two, and three metabolism. It has been reported that thyroid hormone concentrations and activity can be reduced by the induction of xenobiotic metabolizing enzymes, particularly when induction is mediated through the constitutive androstane receptor (CAR). CAR-mediated induction of detoxification enzymes such as Sult2a1 and Ugt1a1 increased T4 clearance and led to decreased T4 and T3 levels (Qatanani et al., 2005). PCBs are CAR activators have been shown on several different occasions to decrease T4/T3 and increase thyroid hypertrophy and TSH (Dean et al., 2002; Gocmen et al., 1989; Hagmar et al., 2001; Koopman-Esseboom et al., 1994; Richardson et al., 2008). Stronger responses have been observed in females (Hagmar et al., 2001), which reportedly have greater CAR levels and activity (Hernandez et al., 2009; Lamba et al., 2003; Ledda-Columbano et al., 2003; Petrick and Klaassen, 2007). Interestingly, the combination of a CAR and
a PPAR\textsubscript{\alpha} agonist can significantly increase thyroid hormone clearance from hepatocytes when compared to only one of the agonists (Wieneke et al., 2009).

117. In contrast, other studies suggest that CAR does not reduce serum T3 concentrations, but instead reduces T3 activity by inducing Dio1 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 (Tien et al., 2007). Therefore, Dio1 induction increases rT3 and in turn represses T3 responsive genes such as tyrosine aminotransferase (TAT), basic transcription element binding protein (BTEB), and carnitine palmitoyl transferase 1 (CPT1) (Tien et al., 2007). 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 (Patrick, 2009).

118. Studies additionally demonstrate that several TDCs directly bind to and/or modify transcriptional activation of TH receptors, including TR\textsubscript{\alpha} and TR\textsubscript{\beta} (Jugan et al., 2010; Kanayama et al., 2005; Patrick, 2009). In vitro binding assays and transactivation assays have been developed to identify thyroid-disrupting chemicals that act as either TR\textsubscript{\alpha}/TR\textsubscript{\beta} agonists or TR\textsubscript{\alpha}/TR\textsubscript{\beta} antagonist ligands. Chemicals with structural similarity to thyroid hormone have been the primary targets of investigation and likely candidates for TR binding (Birnbaum and Staskal, 2004). Kitamura et al., 2005, investigated interaction of tetrabromobisphenol A (TBBPA), a flame retardant, and related compounds using Chinese hamster ovary cell line (CHO-K1) transfected with thyroid hormone receptor TR\textsubscript{\alpha}1 or TR\textsubscript{\beta}1, (Kitamura et al., 2005). In binding assays, several compounds, including TBBPA, tetrachlorobisphenol A (TCBPA), tetrachlorobisphenol A (TCBPA), 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 TR\textsubscript{\alpha}1 or TR\textsubscript{\beta}1. A similar observation has been made with 2,2',4,4'-tetrabromodiphenylether (BDE-47), which exhibits significant thyroid-disrupting activity in mammalian models, but does not exhibit TR binding or receptor transactivation (Suvo et al., 2011). Conversely, in transient transactivation assays TBBPA and TCBPA exhibited significant anti-thyroid hormone effects and appear to function as TR antagonists. Kojima et al., 2009, additionally screened 16 PBDEs and found only 4-OH-BDE-90 displayed antagonist activity (Kojima et al., 2009). BDE206 was also found to inhibit TR-mediated transcription (Schriks et al., 2006). Mechanistically, it is likely that PBDEs/OH-PBDEs affect TH-regulated signal transduction pathways at multiple levels. Recently, however, Ibhazehiebo et al. (2011) propose a mechanism in which the inhibitory activity of several PDBEs is mediated through partial dissociation of TR from TRE cis elements.

119. PCBs additionally suppress thyroid hormone receptor mediated transcription (Iwasaki et al., 2008). A similar mechanism is proposed for PCBs 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 (Miyazaki et al., 2004). This dissociation is observed on both artificial TH-response elements, such as direct repeat (DR)-4, and native TRE-containing promoters, such as malic enzyme (ME)-TRE (Amano et al., 2010). It thus appears that both PBDEs and OH-PCBs may modulate receptor transactivation in a similar fashion.

120. Recent in vitro studies have also demonstrated that dibutyl phthalate monobutyl phthalate (MBP) and di-2-ethylhexyl phthalate exhibit potent TR antagonist activity (Shen et al., 2009). Using a mammalian two-hybrid assay, Shen et al. (2011) demonstrated that 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). The functional significance of this interaction is to be determined, but in some instances, NR interaction with transcriptional co-repressors may lead to enhanced TR regulated gene transcription (Tagami et al., 1997). Other studies have identified additional sites of action in which TR transactivation may be disrupted by TDCs. These mechanisms are detailed in
Juang et al. (2010) and briefly reviewed here (Figure 5-2). 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 co-repressors, such as SMRT or NCOR, repress basal transcription through chromatin deacetylase activity. T3 binding to TR induces 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 (Collingwood et al., 1999; Collingwood et al., 2001).

Thus, in addition to the previously mentioned mechanisms, there are several targets within this process that have been identified as points of action for TDCs (see Figure 5-2). 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; direct binding of TCDs to RXR enabling permissive or non permissive interactions with TR; recruitment; 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 effecting any of the above processes. Theses mechanisms highlight 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.

5.3 Previous HPT Assays- Review by Zoller et al.

In 2007, Zoeller et al., (Zoeller and Tan, 2007) reviewed existing guidelines and strategies for thyroid screening and testing and provided an assessment of existing 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 (Zoeller and Tan, 2007). Table 5-1 provides a listing of HPT assay described in Zoller et al., 2007. While some of these assays have been developed and validated for use by the U.S. Environmental Protection Agency (EPA) and OECD, others were not further assessed for inclusion as validated screening assays.
5.4 **Update on Assays to Detect Disruption of the Thyroid System Across Taxa 2007–2011**

123. 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. Sections are organized as either *in vitro* or *in vivo* assays. Information included in this update represents all current assays and methods currently listed in the published literature between 2008 and 2011.
### Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors

<table>
<thead>
<tr>
<th>Assay</th>
<th>Species</th>
<th>Endpoint</th>
<th>Target effects</th>
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<td>OECD TG 407 Adult Repeated Dose 28-day Oral Toxicity Study</td>
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<td>Total serum T4, TSH, thyroid weight, thyroid histology</td>
<td>Changes in circulating TH levels, hypertrophy or proliferation of thyroid follicles</td>
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<td>OECD TG 414 Prenatal Developmental Toxicity Study</td>
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<td>Changes in circulating TH levels, hypertrophy or proliferation of thyroid follicles, reproductive effects tests</td>
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<td>OECD 415 One Generation Reproductive Toxicity Study</td>
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<td>Amphibian Metamorphosis Assay (21 day)</td>
<td>Xenopus laevis</td>
<td>Hind limb length, thyroid gland histology, whole body length, developmental stage, mortality</td>
<td>Normal, delayed or accelerated metamorphosis from tadpole to frog</td>
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<td>Fish Two Generation</td>
<td>Fathead minnow, medaka, zebrafish, sheeps head minnow</td>
<td>Whole body /serum/tissue T4 levels</td>
<td>Thyroid status</td>
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<td>Avian Two Generation Assay</td>
<td>Japanese quail</td>
<td>Circulating T3 T4, TSH, thyroid weight, histology, bone length, skeletal endpoints, thyroid gland hormone content, body weight/growth rate</td>
<td>Developmental profile of thyroid function, assay of thyroid hormone sensitive tissues (skeleton), HPT axis activation</td>
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<td>Amphibian Growth and Reproduction Test</td>
<td>X. tropicalis</td>
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<td>Normal, delayed or accelerated metamorphosis from tadpole to frog</td>
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<td>Mammalian One or Two Generation Assay</td>
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<td>T3/T4 levels, thyroid histopathology</td>
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<td>Avian Embryo Assay</td>
<td>Japanese quail</td>
<td>Toxicant application to air cell membrane, embryonic and 1 day chick thyroid endpoints including T3/T4 measurements, histopathology, x-ray ray</td>
<td>Developmental endpoint of thyroid function and thyroid hormone action</td>
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<td>Larval Fish Assay</td>
<td>Larval fish</td>
<td>Transition from larval to juvenile form, morphological changes associated with growth, development, hormone content, histopathology</td>
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<td>In vitro Receptor Binding Assay</td>
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<td>T3 receptor binding with isolated THR</td>
<td>Direct THR agonist/antagonist</td>
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<td>THR Transactivation assay</td>
<td>Various cell lines</td>
<td>Functional assay to define pharmacology</td>
<td>Direct THR agonist/antagonist</td>
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<td>Displacement of T4 from proteins</td>
<td>Mechanism by which some chemicals modulate serum T4 levels, may impact T4 uptake into tissues</td>
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<td>Deiodinase</td>
<td>Fish, frog, others</td>
<td>Conversion of T4 to T3 or rT3</td>
<td>Mechanism in which tissues regulate sensitivity to thyroid hormone</td>
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<td>Glucuronidation</td>
<td>Rat, others</td>
<td>T4-glucuronidate</td>
<td>T4 deactivation, reduction in circulating levels of T4</td>
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<td>GH3 Cell Assay</td>
<td>Rat</td>
<td>Growth/proliferation, normal morphology of cells signals, assessment of agonist/antagonist</td>
<td>Local tissue effect of T3</td>
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5.4.1 In Vitro Assays

5.4.1.1 Transient Transfection with TRα and TRβ

124. Numerous studies have employed transient transfection assays to screen compounds for TR agonist and or antagonist activity. The basics of this assay consist of transient expression of TRα or TRβ cloned into a mammalian expression vector (pCDNA, pSG5, or other) containing a strong constitutive promotor 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 promotor containing one or more TR elements (TRE) 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.

125. Transfections using empty vector 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 suggest that the assay is highly sensitive, with an approximate T3-EC₅₀ of 1.50 × 10⁻⁸ M, and maximal induction of 346-fold can be achieved at concentration of 10⁻⁶ M T3 (Shen et al., 2009). Shen et al., 2009 demonstrated that at 10⁻⁶ M T₃ induced luciferase activity with an intra-assay within coefficient of variation (CV) of 7.4% and the inter-assay CV of 18.5% (Shen et al., 2009). 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 (Miyazaki et al., 2004). Antagonistic activity has also been observed with multiple compounds, including OH PCBs, dioxins, and phthalates (Miyazaki et al., 2008; Suvorov et al., 2011). For example, DBP, MBP, and DEHP possessed antagonist activity with IC₅₀ of 1.31 × 10⁻⁵, 2.77 × 10⁻⁶ M and exceeding 1.0 × 10⁻⁵ M, respectively (Shen et al., 2011).

5.4.1.2 Two-hybrid Assays

126. 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 was initially reported by Nishikawa et al., 1999 (Nishikawa et al., 1999). The fundamentals of the assay include development of two fusion proteins, including the yeast GAL4 DNA binding domain (DBD), with the nuclear receptor ligand binding domain (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-NR fusion protein binds to the GAL4 response element within the promoter region of the lacZ gene. Once bound, the GAL4DBD-NR interacts with GAL4AD-co-activator, which recruits the basal transcriptional machinery to the promoter region of lacZ gene, resulting in production of β-galactosidase. β-galactosidase activity 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., 2003 found a lower limit of T3-TR binding activity in this assay to be $3.0 \times 10^8$ M and a calculated EC10 of $1.0 \times 10^6$ M (Kitagawa et al., 2003). 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 through adapting β-galactosidase detection to chemiluminescence (Shiraishi et al., 2003; Terasaki et al., 2011). In an assessment of the thyroid hormone activity of a series of monohydroxy PCBs, Shiraishi et al. (2003) incorporated rat liver S9 fraction in the yeast two-hybrid assay to determine necessity of metabolic activation prior to TR binding. Chemicals are first incubated with rat liver S9 fraction, followed by addition of yeast to the assay system. Similarly, Li et al. (2008) developed a yeast two-hybrid assay using the human TRβ/GRIP coactivator system. TDC antagonist activity was assessed in the presence of $5.0 \times 10^6$ M T3, which induced maximal β-galactosidase activity. Results of this study identified two partial TRβ agonists, including 2-ethylbutylphenol and 2-isopropylphenol. The remainder of compounds screened exhibited partial antagonist activity, with 20% relative inhibitory concentration (RIC20) greater than $10^7$ M; however, PHAHs exhibited RIC20 values less than $5 \times 10^7$ M following incubation of rat liver S9 fraction. More recently, Terasaki et al. (2011) demonstrated that halogenated derivatives of bisphenol A (BPA), 3,30,5,50-tetrabromobisphenol A (TBBPA), 3,30,5,50-tetrachlorobisphenol A (TCBPA), and 3,30,5-trichlorobisphenol A (3,30,5-triClBPA) exhibit 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 TR alpha at $2 \times 10^5$ M without rat liver S9 treatment and $4 \times 10^6$ M with rat liver S9 treatment, demonstrating their T3 antagonist activity.

127. Mammalian two-hybrid systems have been useful to screen nuclear receptor-nuclear receptor co-regulator protein-protein 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 the interaction domain (LXXLL) of a nuclear receptor co-regulator (coactivator or corepressor), VP16-hTR fusion protein, and a Gal4 responsive luciferase reporter such as pUAS-luc. Following transfection, cells are treated with compounds of interest and examined for ligand-dependent recruitment of nuclear receptor and coregulator interactions. The relative transcriptional activity is converted to fold induction above the corresponding vehicle control value (n-fold).

128. Based upon data generated using 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 TCDs can either facilitate or modulate coregulator (coactivator and/or corepressor) interaction with TR. Investigations into the mechanisms of di-n-butyl phthalate (DBP) and mono-n-butyl phthalate (MBP), Shen et al., 2011 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 (Shen et al., 2011). Conversely, Ibhazehiebo et al., 2011 found that polybrominated biphenyl mixture BP-6 did not recruit corepressors to TR or inhibit coactivator binding to TR in the presence of ligand (Ibhazehiebo et al., 2011). Similarly, PBDEs did not alter ligand-dependent cofactor (SRC-1) recruitment to TRβ1 (Ibhazehiebo et al., 2011).

5.4.1.3 DNA Binding Assays

129. 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 (Iwasaki et al., 2008). 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 glutathionemophorase beads and incubated with a digoxigenin (DIG)-labeled double-stranded DNA fragment containing a TRE. After repeated washing, protein–DNA binding on sepharose beads is detected using anti-DIG antibody conjugated to alkaline phosphatase, which is then measured by a chemiluminescent reaction using a luminometer. Using this approach Ibhazehiebo et al., 2011 discovered that repression in transactivation of TR following exposure to polybrominated biphenyls and PBDEs is due to partial dissociation of TR from TH response element (TRE) (Ibhazehiebo et al., 2011).

5.4.1.4 Dendritic Arborization

130. 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 via TR gene transactivation (Bradley et al., 1992; Strait et al., 1991). 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 Purkinje cells, as described by Kimura-Kuroda et al. (2005), 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. (2005) 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 bisphenol A 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 (Kimura-Kuroda et al., 2007, 2008). More recently, Ibhazehiebo et al., 2011 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 (IbhazehieboIwasakiKimura-Kuroda et al., 2011; IbhazehieboIwasakiOkano-Uchida et al., 2011).

5.4.1.5 Neurite Extension

131. As with Purkinje cells, TRs are ubiquitously expressed in most cerebellar neuronal cells, including granule cells during development (Bradley et al., 1992). Cerebellar granule cell have been used extensively as a model system for studies on mammalian central nervous system neurogenesis (Nishikawa et al., 1999). 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., 2004, 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 (Okano-Uchida et al., 2011; Okano-Uchida et al., 2004). 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 (Xu et al., 2011). 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, Ibhaezhiebo et al., 2011 demonstrate 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 (Okano-Uchida et al., 2011).

**5.4.1.6 Cell Proliferation Assay**

132. The “T-screen” is a cell assay proliferation based assay used for the *in vitro* detection of TR agonists and antagonists (Gutleb et al., 2005). 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 (O'Brien et al., 2000). 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%).

133. 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 cheap 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 multiple compounds, including PCBs (Gutleb et al., 2007), PAHs (SchriksVrabie et al., 2006), nitrates (Hansen et al., 2009), and others. Additional modifications to the T-screen include incorporation of a metabolic system to the assay. Taxvig et al., 2011 tested both the human liver S9 mix and the PCB-induced rat 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 and assess the endogenous metabolic capacity of the GH3 cells (Taxvig et al., 2011). 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 the 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.

134. Schreiber et al. (2010) 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 preincubated period with test compounds. Cell proliferation is determined by measuring sphere size. Migration is measured though determining the distance from the edge of the sphere to the farthest 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.
5.4.1.8 Thyroid Peroxidase Inhibition Assay

135. Thyroid Peroxidase (TPO) is a heme protein localized in the apical cytoplasmic membrane of thyroid epithelial cells and plays an important role in thyroid hormone biosynthesis (Zoeller et al., 2007). 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 (Gotthardt et al., 2007). Schmutzler et al., 2007 developed a novel in vitro assay based on human recombinant TPO (hrTPO) stably transfected into the human follicular thyroid carcinoma cell line FTC-238 (Bacinski et al., 2007). 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 assed in vitro for peroxidase activity using the guaiacol oxidation assay, as previously described (Hosoya, 1963). TPO activities are calculated as micromole H₂O₂ 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.

136. 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 (4-NP) and BPA also inhibited TPO, with IC₅₀ values ranging from 0.83 to 174 μmol/L, where, as compounds, including 4-MBC, procymidon, linuron, BP3, 4-NP, and estradiol, had no effect on TPO activity (Gotthardt et al., 2007).

5.4.1.9 Iodide Uptake Assay

137. 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 (Gotthardt et al., 2007). 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 ¹²⁵I containing media 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 and 4-MBC, and industrial compounds, including 4-NP and perchlorate (Gotthardt et al., 2007). One compound tested, xanthohumol exhibited stimulation of iodide uptake by NIS at nanomolar concentrations.

5.4.1.10 Thyroid Hormone Binding Protein Assays

138. Several studies have shown that in vivo exposure of experimental animals to TDCs results in reduction of the thyroid hormone thyroxine (T₄) level in serum due to binding with thyroid hormone transport proteins and displacement of T₄ (Meerts et al., 2000). To investigate the binding interactions of TDCs with hormone-binding proteins, Cao et al., 2010, utilized a novel fluorescence displacement method (Cao et al., 2010). 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 IC₅₀ value can be obtained, and the binding constant of the analyte with the protein can be calculated. In this assay, 8-anilino-1-napthalenesulfonic acid (ANSA) is used as the fluorescence probe due to its know iteration 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 × 10⁷ M⁻¹ and 6.9 × 10⁶ M⁻¹ for TTR and between 6.5 × 10⁶ M⁻¹ and 2.2 × 10⁸ M⁻¹ for TBG.
5.4.2 In Vivo Assays

5.4.2.1 Modification of Long-term in Vivo Assays

139. Multiple long term in vivo bioassay methods that include thyroid-related endpoints have been developed in a variety of species, including rat (OECD TG 407: Adult Repeated Dose 28-day Oral Toxicity Study or OECD 416: Two Generation Reproductive Toxicity Study and others), fish (Fish Two Generation), and amphibians (Amphibian Metamorphosis Assay (21 day), (for a complete list, see those previously described by Zoller et al., 2007) (Zoeller and Tan, 2007). 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 (Zoeller and Tan, 2007). 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 in vivo assays and compared to results obtained in long-term studies.

140. Tietge et al., 2010 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 (100mg/L), 6-propylthiouracil (6-PTU) (20mg/L), and perchlorate (4 mg/L) (Tietge et al., 2010). 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 (ETU) 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 (Yamada et al., 2011).

141. 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., 2011 recently examined gene expression differences for target genes, including BTEB, TRβ, BDNF, GAP-43, and NCAM1 in of rat brain following gestational exposure to perfluorooctane sulfonate (PFOS) and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) on PNDs 1, 7, and 14 (Liu et al., 2011).

142. Gene expression changes have also been assessed in non-mammalian models, including Xenopus and zebrafish. For example Shen et al., 2001 reported gene targets, including TRβ, RXRγ, and TSHα and TSHβ, were each modified following exposure to DBP and MBP in Xenopus laevis (Shen et al., 2011). Similarly, the chemical-induced effects impacting cross-talk between the hypothalamic–pituitary–gonad (HPG), HPA, and HPT axes of prochloraz (PCZ) or propylthiouracil (PTU) exposed adult zebrafish was examined using 20 gene qPCR array (Bradley et al., 1994).

143. Multiple studies have additionally applied a microarray and other transcriptomic approaches to assess global gene expression changes following TDC exposures in vivo. Heimeier et al., 2010, used a microarray approach to anchor BPA-induced gene expression changes with intestinal remodeling in premetamorphic Xenopus laevis tadpoles (Bradley et al., 1992). Importantly, microarray analysis revealed that BPA antagonized the regulation of most T(3)-response genes, thereby explaining the inhibitory effect of BPA on metamorphosis. Similarly, Ishihara et al., 2011, used gene expression profiling to examine the thyroid hormone-disrupting activity of hydroxylated PCBs in metamorphosing amphibian tadpole (Ishihara et al., 2011). Results form this study 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 mechanism underlying thyroid-disrupting activities in vivo.

5-15
5.4.2.2 Additional In Vivo Models

144. 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 (Raldua and Babin, 2009). 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 bases of the assay examines the impact of TDC to abolished T4 immunoreactivity in thyroid follicles of zebrafish larvae.

145. Transgenic reporter animals additionally have the potential to be incorporated into in vivo TDC screening protocols. Terrien et al., 2011, 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 laevis TH/bZIP promoter. Exposure of this line to thyroid hormone (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 (1). In 2007, Fini et al., reported development of a high-throughput method to assess potential effects of endocrine disrupting chemicals (EDCs) in Xenopus in vivo (Fini et al., 2007). 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 a 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.

Organ culture (Ex vivo)

146. 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 develop assays to directly investigate chemicals for thyroid hormone disruption using thyroid gland explant cultures from X. laevis tadpoles (Hornung et al., 2010). 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 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.

147. A similar approach was used by Schriks et al., 2006, where a X. laevis tadpole tail tip regression assay was used as a bioassay to detect thyroid hormone disruption (Schriks et al., 2006). The basis of this assay stems form 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 to selected chemical agents in the presence of 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 (Davey et al., 2008).

### 5.5 Challenges and Limitations.

148. 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 assays 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 TCDs. 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 high-throughput capacity, enabling large-scale screening for this mechanism. Thus, several newly developed assay 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. 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.

149. It is likely that any screening process for thyroid hormone disruption will incorporate a battery of both *in vivo* and *in vitro* assays. The above description provides a basis to assess how to best approach developing an appropriate compliment of assays. High-throughput screens will likely incorporate both *in vitro* and a few *in vivo* assays that are amenable to scale up. Validation of HPT disruption is likely to be conducted using longer-term *in vivo* assays.
6. The Vitamin D Signaling Pathway

6.1. Overview of the Vitamin D Signaling Pathway

6.1.1. Synthesis

150. All vertebrates possess the vitamin D endocrine axis (Lock et al., 2010). 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 D₃ 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 (Bouillon et al., 2008; Norman, 2008). 7-dehydrocholesterol absorbs UVB light in the 290–315 nm wavelength, which breaks the bond between carbons 9 and 10, creating pre-vitamin D₃. Pre-vitamin D₃ is thermodynamically unstable and rapidly isomerizes to vitamin D₃ (Dusso et al., 2005; Holick et al., 1977). 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 D₃ production in the skin (Holick and Chen, 2008). Vitamin D₃ is not biologically active and must be metabolized to its active form through two hydroxylation reactions.

151. The first hydroxylation reaction takes place in the liver. Vitamin D₃ is transported from the skin bound to transport proteins in the bloodstream. Most vitamin D₃ is bound to vitamin D binding protein (DBP), but some is also bound to albumin (Christakos et al., 2003; Dusso et al., 2005). Once in the liver, the P450 enzyme 25-hydroxylase (CYP2R1) adds a hydroxyl group to carbon 25, creating 25-hydroxyvitamin D₃. This hydroxylation step is not well regulated and is dependent on vitamin D₃ substrate availability. Because this reaction reflects the vitamin D₃ status of an individual, measuring serum levels of 25-hydroxyvitamin D₃, it is a common method of determining the vitamin D status of patients (Dusso et al., 2005). Although multiple P450 enzymes are capable of hydroxylating vitamin D₃ at C25, evidence indicates that CYP2R1 is the most likely candidate (Schuster, 2010). Genetic defects in CYP2R1 cause a type of hereditary rickets, while defects in other candidate P450 enzymes do not affect circulating levels of 25-hydroxyvitamin D₃ (Prosser and Jones, 2004; Schuster, 2010).

152. After the initial hydroxylation, 25-hydroxyvitamin D₃ is once again bound to transport proteins and transported in the blood to the kidney for the second hydroxylation reaction. The 25-hydroxyvitamin D₃-DBP complex is filtered out of the blood by the glomerulus is absorbed at the proximal tubules of the kidney by endocytosis mediated by a surface receptor protein called megalin (Nykjaer et al., 1999). Megalin-deficient mice are unable to reabsorb 25-hydroxyvitamin D₃ at the proximal tubules and instead excrete the vitamin D₃ metabolite in their urine. These mice suffer from vitamin D deficiency and rickets (Nykjaer et al., 1999). Once inside the cells of the proximal tubules, DBP is degraded while 25-hydroxyvitamin D₃ 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 D₃, creating 1α, 25-dihydroxyvitamin D₃, which is the active metabolite of vitamin D₃ (Dusso et al., 2005).

153. Unlike the first hydroxylation, the second hydroxylation is tightly regulated. This regulation is necessary in order to maintain proper 1α, 25-dihydroxyvitamin D₃ levels. One of the major roles for vitamin D in vertebrates is calcium homeostasis through the control of calcium absorption in the intestine, and the 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 D₃ that
causes an increase in serum calcium concentration (Omdahl et al., 2002; Schuster, 2010). Mutations in 1α-hydroxylase cause vitamin D-resistant rickets type 1 in humans.

### 6.1.2 Catabolism

154. 1α, 25-dihydroxyvitamin D₃ 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 D₃ to calcitroic acid through a series of hydroxylations and side chain oxidations (Prosser and Jones, 2004). The breakdown products are eliminated from the body. Nearly all cells in the body express 24-hydroxylase, but the highest activity is seen in the kidney (Omdahl et al., 2002). Mice lacking CYP24A1 can not clear 1α, 25-dihydroxyvitamin D₃ from their bloodstream, and the active form of vitamin D remains in their bloodstream for days (St-Arnaud et al., 2000). 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.

### 6.2 Biological Functions of the Vitamin D Signaling Pathway

155. 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 D₃ to its hormone receptor, the 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 D₃ binds to the ligand-binding pocket of VDR with high affinity (Kd = 10⁻¹⁰ to 10⁻¹¹) (Dusso et al., 2005). 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 (Norman, 2008). Co-regulatory proteins are recruited, followed by the recruitment of RNA polymerase II and the initiation of gene transcription.

156. 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 (Owen and Zelent, 2000). 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 (Reschly and Krasowski, 2006; Whitfield et al., 2003). 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 (Reschly et al., 2007). In humans and rodents, thirty-six tissues express VDR, including tissues that are not associated with the classic vitamin D effects of calcium mobilization and ion homeostasis (Norman, 2008). 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 6-1).

### 6.2.1 Calcium and Skeletal Maintenance

157. 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 (Clarke, 2008). Vitamin D regulates many actions of osteoblasts, including cell proliferation, bone matrix synthesis, mineralization, and the initiation of osteoclastogenesis (Nagpal et al., 2005; van Driel et al., 2004).

158. Vitamin D and VDR are both necessary the expression of transport channels and proteins necessary for proper calcium absorption from the small intestine (Dusso et al., 2005). 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.

### 6.2.2 Immune System Function

VDR is widely expressed in multiple immune cells, including T lymphocytes, macrophages, and dendritic cells (Mathieu et al., 2004; Veldman, 2000). Immune cells are capable of producing and maintaining local concentrations of 1α, 25-dihydroxyvitamin D₃ through the expression of both 1α-hydroxylase and 24-hydroxylase (reviewed in (Bouillon et al., 2008)). 1α, 25-dihydroxyvitamin D₃ 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 (Compston and Coles, 2008; Danescu et al., 2009; Hanwell and Banwell, 2010; Nagpal et al., 2005). Vitamin D plays a role in 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 (Mathieu et al., 2004).

Vitamin D is either synthesized in the skin or obtained through the diet. Vitamin D₃ is transported to the liver and undergoes the first hydroxylation reaction by 25-hydroxylase, creating 25-hydroxyvitamin D₃. This compound is transported to the kidneys for the second hydroxylation by 1α-hydroxylase to create the active metabolite: 1α, 25-hydroxyvitamin D₃. 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 vitamin D receptor. The list of tissues for this diagram was taken from Table 1 in (Norman, 2008).
6.2.3 Cancer

160. VDR and vitamin D status has 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 (Nagpal et al., 2005). 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 (Dusso et al., 2005; Nagpal et al., 2005).

6.2.4 Neurodevelopment

161. Vitamin D is an important neurosteroid with critical roles in vertebrate brain development (Cannell, 2008; Humble, 2010). The vitamin D receptor and P450 enzymes involved in vitamin D synthesis and catabolism are expressed in the brain, central nervous system, and peripheral nervous system (Dusso et al., 2005; Nagpal et al., 2005). 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 (Cannell, 2008; Humble, 2010). 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 and autism (Cannell, 2008; Humble, 2010).

6.2.5 Cardiac Function

162. 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 D3. The renin-angiotensin system directly regulates blood pressure and salt/water homeostasis. The gene for renin contains a VDRE in the promoter, and it has been shown that 1α, 25-dihydroxyvitamin D3-bound VDR directly inhibits renin expression. Renin is a protease that cleaves angiotensinogen to angiotensin I, which is then converted to angiotensin II. Angiotensin II levels regulate blood pressure, volume, and electrolyte homeostasis (Bouillon et al., 2008; Nagpal et al., 2005). 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 (Vanga et al., 2010). There is a strong correlation between vitamin D deficiency and multiple cardiovascular diseases, including hypertension, coronary artery disease, and heart failure (Vanga et al., 2010).

6.3 Vitamin D Disrupting Chemicals and Effects

6.3.1 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

163. The vitamin D receptor maintains high substrate fidelity; thus, few EDCs are likely to interact with this receptor directly (Reschly and Krasowski, 2006). However, studies in laboratory animals and wildlife exposed to dioxins (such as TCDD) and dioxin-like compounds have shown altered vitamin D3 serum levels and associated bone malformations (Alvarez-Lloret et al., 2009; Haynes et al., 2009; Lilienthal et al., 2000; Nishimura et al., 2009; Routti et al., 2008). Although this evidence suggests that dioxins may be disrupting the vitamin D endocrine system, it is fairly clear that dioxins are not VDR ligands (Reschly et al., 2007). Dioxins are ubiquitous and persistent environmental contaminants and potent endocrine disruptors in multiple biological systems. Effects of dioxin exposure include reduced reproductive success, decreased survival of early life stages, and perturbations in growth and development (Jobling and Tyler, 2003). Classic signs of TCDD toxicity in teleosts include alterations in cardiovascular
development and function, craniofacial malformations, delayed growth, and death (Dong et al., 2010; Jobling and Tyler, 2003). Effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR) in vertebrates. Xenobiotic detoxification is the classic role of AhR. It is also thought to be associated with organogenesis and development (Bock and Koechle, 2006; Gu et al., 2000). Like VDR, AhR is expressed in both osteoblasts and osteoclasts (Ilvesaro et al., 2005; Ryan et al., 2007). TCDD has been shown to inhibit osteoblast differentiation and osteoclastogenesis, but the mechanism(s) of action remain unknown (Korkalainen et al., 2009; Ryan et al., 2007). 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 (Matsunawa et al., 2009; Nishimura et al., 2009).

164. Our 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 (Lilienthal et al., 2000). Lake Ontario is highly contaminated with dioxins, and minks consuming fish from the lake have a specific jaw lesion that is considered a biomarker for TCDD exposure (Haynes et al., 2009). 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.

6.3.2 Polychlorinated Biphenyls (PCBs)

165. Polychlorinated biphenyls (PCBs) are persistent organic pollutants that were commonly used as coolants and insulators in capacitors and transformers. PCBs are currently banned in the United States, but they still persist in the environment. Exposure to PCBs has been shown to alter bone homeostasis, strength, and composition. Few studies exist that assess PCB effects on vitamin D₃. Alvarez-Lloret et al. (Alvarez-Lloret et al., 2009) found decreased serum vitamin D₃ levels and decreased bone mineralization and composition in rats exposed to PCB126. Lilienthal et al. (Lilienthal et al., 2000) also noted decreased serum vitamin D₃ levels in rats exposed to a PCB mixture. Bone lesions were observed in seals that had been exposed to high PCB and DDT levels. The investigators speculated that these effects were related to a disruption of the vitamin D and thyroid hormone signaling (Routti et al., 2008). The exact mechanism of action of PCB disruption of the vitamin D endocrine system is unknown and requires further investigation.

6.3.3 Secondary Bile Acids

166. Bile acids are end products of cholesterol metabolism that play an important role in the intestinal absorption of lipids (Hagey et al., 2010). 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.

167. 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 refers to bile acids that are synthesized from cholesterol in the hepatocyte of the liver (Hofmann and Hagey, 2008). 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 (Makishima et al., 2002). There is a positive correlation among high-fat diets, increased LCA concentrations, and colon cancer.
168. 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 (Jurutka et al., 2005; Matsubara et al., 2008). 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 (Makishima et al., 2002). VDR-mediated protection against colon cancer is decreased in situations causing vitamin D deficiency or in high-fat diets. The highest death rates from colon cancer occur in areas with a high prevalence of rickets (Makishima et al., 2002).

169. 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 and in-depth review of vertebrate bile acids and alcohols, see (Hagey et al., 2010) and (Hofmann et al., 2010).

6.3.4 Ethanol

170. Chronic alcohol consumption can alter bone growth and remodeling, resulting in decreased bone density and an increased risk of bone fractures (Sampson, 1998). Studies in rats have shown that chronic alcohol consumption results in reduced serum 1α, 25-dihydroxyvitamin D3 levels as a result of both decreased CYP27B1 and increased CYP24A1 expression (Shankar et al., 2008). Other studies have shown similar results in rats and chickens (Kent et al., 1979; Peng et al., 1990).

6.3.5 Lead

171. 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 (Dusso et al., 2005). Three VDR polymorphisms—BsmI, Apal, FokI (named for their identifying restriction sites)—have been shown to affect lead concentrations in whole blood and plasma (Rezende et al., 2008). Lead accumulates in bone tissue during bone growth and remodeling and has been shown to compete with calcium for common transport mechanisms (Fullmer, 1997). 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 (Rezende et al., 2008). 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 as a result of 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 individuals with all three of these polymorphisms may have a protective effect on the fetus against lead exposure (Rezende et al., 2010). Although these VDR polymorphisms may have a protective effect against lead toxicity, it is important to remember that they may be less functional and could cause other health consequences related to the vitamin D endocrine system.

6.4 Screening Assays

172. There are very few studies that have evaluated the effects of contaminants on the vitamin D signaling pathway. All of these studies were performed with mammals or mammalian cells. Common methods used in these studies are summarized below, followed by a section of suggested methods for future studies.

6.4.1 Serum Vitamin D Levels

173. Analyses of circulating vitamin D or its metabolites may be a viable endpoint when assessing the effects of chemicals on this signaling pathway. There are multiple methods of measuring
serum vitamin D concentrations (see Wallace et al., 2010, for a thorough review of the types of assays). The most common method for measuring serum vitamin D₃ levels is by radioimmunoassay (RIA). Commercially available kits can be used to detect vitamin D₃ or specific metabolites by RIA.

174. Routti et al. (2008) used a radioimmunoassay from Diasorin (Stillwater, MN) to determine circulating levels of 1α,25-dihydroxyvitamin D₃ in seals exposed to DDT and PCBs. Shankar et al. (2008) used kits from the same company to measure changes in both serum 25-hydroxyvitamin D₃ and 1α, 25-dihydroxyvitamin D₃ levels in rats after long-term ethanol exposure. Nishimura et al. (2009) used a non-radioactive enzyme-linked method from Immunodiagnostic Systems to determine serum 1α,25-dihydroxyvitamin D₃ levels in mice exposed to TCDD. All of the kits used in these studies were sensitive enough to show significant decreases in serum vitamin D levels in exposed populations.

### 6.4.2 Changes in Expression of Vitamin D-Responsive Gene Products

175. The assays described above would inform a researcher whether or not serum vitamin D concentrations have changed, but additional assays would be needed to assess the cause of these changes, or the impacts of altered vitamin D levels. Evaluating changes in the expression of vitamin D related genes is one way to accomplish this. Quantitative polymerase chain reaction (qPCR) can be used for detecting changes in the expression of genes involved in the vitamin D signaling.

176. Nishimura et al. (2009) used qPCR to quantify expression changes for twenty vitamin D related genes in C57BL/6J mice after exposure to TCDD. They evaluated genes involved in vitamin D metabolism, transport, catabolism, and biomarkers for vitamin D gene targets in the intestine and bone. Shankar et al. (2008) used qPCR assess expression changes in P450 enzymes involved in the synthesis and metabolism of vitamin D in Sprague-Dawley rats after exposure to ethanol. They found that renal CYP27B1 expression was decreased and CYP24A1 expression was increased, which could explain the observed decreased serum vitamin D concentrations.

### 6.4.3 Skeletal Morphology

177. Although the vitamin D endocrine system has an effect on numerous tissue types, most studies have focused upon the effects of endocrine disruptors on bone, the classic target tissue of vitamin D. Skeletal abnormalities are often accompanied by changes in vitamin D status. Alterations to skeletal integrity are examined by measuring changes in various bone characteristics, such as bone mineral density (BMD), bone mineral content (BMC), bone thickness (BT), mechanical strength, changes in cell content, and gross changes in skeletal structure.

178. Nishimura et al. (2009) and Finnilä et al. (2010) examined the tibias of mice exposed to TCDD for changes in bone characteristics, while Alvarez-Lloret et al. (2009) used lumbar vertebra in PCB126 exposed Sprague-Dawley rats. All three groups measured BMD, BMC, and BT, but Finnilä’s group also measured cross-sectional area. Nishimura’s group made their measurements using dual energy X-ray absorptiometric analysis, while Finnilä’s 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 BMD, BT, and BMC. It should be noted that Finnilä’s group did not measure vitamin D levels in the mice.

### 6.4.4 Histology

179. Histology is a method to study microscopic changes in cell and tissue structure. 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 and tartrate-resistant acid phosphatase (TRAP) to stain bone-resorbing osteoclasts; and Villaneuva’s Goldner to differentiate between mineralized and unmineralized bone.

180. Histology staining is a common method used to evaluate the effects of contaminants on bone, but it is not often reported in studies that have also analyze vitamin D levels. Nishimura et al. (2009) stained tibia sections from 21-day-old TCDD exposed mice with Villanueva’s Goldner 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 since TCDD is known to induce osteoclastic bone formation.

6.4.5 Immunohistochemistry

181. Immunohistochemistry also involves the use of tissues mounted onto slides, only in this case, antibodies are used to assess the distribution and tissue localization of a protein. Nishimura et al. (2009) used immunohistochemistry to visualize calbindin-D28K and 1α-hydroxylase proteins in the kidneys of mice exposed to TCDD and to visualize calbindin-D28K in the small intestine and PTH in the parathyroid gland.

6.5 Current Challenges and Limitations

6.5.1 Limited Knowledge Regarding Non-mammalian Vertebrates

182. 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 vitamin D receptor as a result of a whole-genome duplication event specific to the teleost lineage. Mammals and other vertebrates only have one copy (Howarth et al., 2008; Meyer and Van de Peer, 2005). 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 early VDR may have additional functions other then calcium mobilization (Whitfield et al., 2003). Studies in Xenopus have shown that VDR is expressed before bone formation takes places (Li, 1997). Howarth et al. (2008) has shown 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. The Peroxisome Proliferator-Activated Receptor Signaling Pathway

7.1 Overview of PPAR Signaling Pathway

183. 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. 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 (Amoutzias et al., 2007).

184. The LBD of the PPARs is large, with a binding pocket of approximately 1300–1400 Å², and can accommodate large ligands such as fatty acids. The LBD for PPARα and PPARγ are quite similar and show affinity for similar ligands. The greater lipophilicity of the binding pocket of PPARα may account for its higher affinity for saturated fatty acids. A one amino acid difference accounts for the increased pharmacological sensitivity of PPARγ for the thiazolidinedione drugs (Xu et al., 2001). In contrast, PPARδ has a large LBD, but its pocket is much more narrow (Xu et al., 2001).

185. 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 (Abbott, 2009; Pyper et al., 2010). PPARα controls β-oxidation in the peroxisomes and mitochondria, and ω-oxidation in the microsomes of the liver. In turn, PPAR ligands reduce VLDL, increase HDL, and reduce the duration of macrophage-induced inflammation (Devchand et al., 1996; Lefebvre et al., 2006). 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 (Girroir et al., 2008). PPARβ/δ activation also improves fatty acid catabolism in skeletal muscle (Barish et al., 2006; Grimaldi, 2007). 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 (Willson et al., 2001).

7.1.1 PPARs in Non-Mammalian Species

186. PPARs have been detected in chicken, Xenopus, and several fish species. The fact that peroxisome proliferation is mediated through PPARs was first discovered in Xenopus (Dreyer et al.,...
1992), and three PPARs have been identified in *Xenopus*, PPARα, PPARβ/δ, and PPARγ (Desvergne and Wahli, 1999). The chicken genome also contains all three PPAR members with similar expression profiles as mammals (Hojo et al., 2006; Meng et al., 2004). Studies indicate the presence of four PPARs in the Japanese pufferfish (*Fugu*) genome: two PPARαs, PPARδ, and PPARγ (Maglich et al., 2003). Interestingly, zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) also express two distinct PPARβ/δ genes (Leaver et al., 2005; Robinson-Rechavi et al., 2001). The four PPARs in *Fugu* show wide tissue distribution, whereas in mammals, only PPARδ is widely distributed. Sea bream (*Sparus aurata*), plaice (*Pleuronectes platessa*) (Leaver et al., 2005), and sea bass (*Dicentrarchus labrax*) also express PPARα, PPARβ/δ, and PPARγ. However, these studies indicate that PPARα and PPARδ demonstrate similar tissue distribution to mammals, while PPARγ showed wide tissue distribution. PPARα, β, and γ have also been detected in the liver of gray mullet (*Mugil cephalus*) and zebrafish using mouse antibodies, which may make fish sensitive to the effects of peroxisomal proliferators (Ibabe et al., 2002; Ibabe et al., 2004). *Table 7-1* lists the function of each PPAR.

<table>
<thead>
<tr>
<th>PPAR</th>
<th>Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferation, liver cancer, fatty acid metabolism, lipid homeostasis, inflammation, development</td>
</tr>
<tr>
<td>PPARβ/δ</td>
<td>Fatty acid metabolism, skin proliferation, placental development</td>
</tr>
<tr>
<td>PPARγ</td>
<td>adipocyte differentiation, glucose homeostasis, represses inflammation, placental development</td>
</tr>
</tbody>
</table>

### 7.2 Precedent Chemicals

187. 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 (Desvergne and Wahli, 1999). 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 (Desvergne and Wahli, 1999; Forman et al., 1997; Kliwer et al., 1997). Recent evidence indicates that the endogenous PPARα ligand in the liver is 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (Chakravarthy et al., 2009). Other endogenous PPAR ligands include ceramides that indirectly activate PPARδ (Jiang et al., 2009). Leukotrienes and prostaglandins are also activators of PPARs, where leukotriene B4 activates PPARα but PGJ2 does not (Lin et al., 1999), while PGJ2 preferentially activates PPARγ (Raman et al., 2011; Waku et al., 2009) (*Table 7-2*).

188. The xenobiotic ligands of PPARs include the hypolipidemic drugs and PPARα activators such as Wy 14,463, clofibrate, ciprofibrate, methylclofenapate, clobufariz, fenofibrate, and fosexafen. 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 (Abbott, 2009; Forman et al., 1997; Kliwer et al., 1997) (*Table 7-2*). Several plant extracts have recently been shown to activate all three PPARs. This includes the carnosic acid and carnosol found in sage and rosemary, which activates PPARγ (Rau et al., 2006). In fact, almost 50% of the plant extracts tested showed activation of PPARγ, and a little more than 25% tested showed activation of PPARα (Rau et al., 2006); however, the physiological significance of this activation is not known.

189. 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 (Lampen et al., 2003), including PPARα activation by monobenzylphthalate, mono-butylylphthalate, and mono-2-ethylhexylphthalate (MEHP) (Hurst and Waxman, 2003). MEHP and 2-ethylhexanoic acid (EHA) that are metabolites of the commonly used phthalate, di-ethylhexylphthalate, activate PPARs with EHA showing a preference for PPARα and MEHP demonstrating similar activity towards both PPARα and PPARγ (Maloney and Waxman, 1999). However, given that DEHP and MEHP are lipophilic, PPARγ, which is localized in adipose tissue, may be the most physiologically relevant PPAR target (Grun and Blumberg, 2009; Maloney and Waxman, 1999).

190. Perfluorooyctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) activate PPARα. The developmental defects caused by PFOA are lost in PPARα-null mice (Abbott et al., 2007), but the defects caused by PFOS are not lost in PPARα-null mice (Abbott et al., 2008). Perfluorochemicals also activate Baikal Seal (Pusa Sibirica) in PPARα (Ishibashi et al., 2011); however, whether there are consequences on development or peroxisome proliferation is not known. There is also evidence that PFOA activates PPARγ (Botts, 2007; Rosen et al., 2008; Vanden Heuvel et al., 2006), but some laboratories have not been able to verify this result (Takacs and Abbott, 2007).

191. Trichloro- and dichloroacetic acid, metabolites of trichloroethylene, are PPARα activators (Maloney and Waxman, 1999), and the herbicide Dicamba (2-methoxy-3,6-dichlorobenzoic acid) is another suspected PPAR ligand (Espandiari et al., 1995). 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 PPARγ (Maloney and Waxman, 1999).

192. The persistent organotins such as tributyltin and triphenyltin that are used as antifouling agents, wood preservatives, and in polyvinylchloride plastics are also PPARγ agonists (Grun et al., 2006; Kanayama et al., 2005). Interestingly, the organotins also activate PPARs’ requisite heterodimeric partner, RXR (Section 5). Organotins induce weight gain, fat mass, and adipocyte number (Grun et al., 2006; Grun and Blumberg, 2009; Kanayama et al., 2005), and it has been hypothesized that the dual action of organotins on both RXR and PPARγ enhance organotin’s actions (Grun and Blumberg, 2009) because RXR ligands increase PPAR activity (Feige et al., 2006). Transactivation assays performed with rainbow trout (Oncorhynchus mykiss) PPARs provide support for the idea that RXR activation enhances PPAR activity as bezafibrate activates PPARβ at 50 nM concentrations, but co-treatment with 9-cis retinoic acid enhanced assay sensitivity so that concentrations nearly 10 times lower (6 nM bezafibrate) significantly activate PPARβ (Liu et al., 2005).

193. The PPARs of Xenopus are activated by many of the same chemicals that activate human PPARs although PPARβ from Xenopus is much more sensitive to bezafibrate than PPARβ from mammals (Desvergne and Wahli, 1999). Direct activation of Xenopus PPARs by environmental chemicals such as tributyltin, PFOA, and phthalates has not been evaluated. However, TBT increases ectopic adipocyte formation around the gonads in mice and Xenopus laevis, indicating that TBT activates Xenopus PPARγ in vivo (Grun et al., 2006). 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 (Oehlmann et al., 2009).

194. PPARγ from fish species show significantly different activation profiles than the human ortholog. Fugu PPARγ only has two hydrogen bonding residues in its ligand binding pocket and is unlikely to bind fatty acids with high affinity (Maglitch et al., 2003). Transactivation assays confirm that PPARγ from sea bream or plaice is not activated by fatty acids or typical mammalian PPARγ synthetic ligands (Leaver et al., 2005). Interestingly, proteomic analysis of arsenic-exposed zebrafish indicates that
arsenic activates PPAR\(\gamma\) pathways in male zebrafish (Carlson et al., 2011). Some have speculated that arsenic may be associated with increased risk of diabetes in humans (Sharp, 2009), providing evidence of an important role for comparative studies in human health. Overall, fish PPARs are complex (Leaver et al., 2007), and evolutionary differences between fish and human indicate that data from mammalian species may not project to keystone environmental species and vice versa.

### Table 7-2. Examples of PPAR Activators

<table>
<thead>
<tr>
<th>Chemical</th>
<th>PPAR(\alpha)</th>
<th>PPAR(\beta/\delta)</th>
<th>PPAR(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Saturated fatty acids (C14:0 – C18:0)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(\omega)-3 unsaturated fatty acids (C18:22)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(\omega)-6 unsaturated fatty acids (C18:20)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>(\omega)-9 unsaturated fatty acids (C16:18)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Prostaglandin J2</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Leukotriene B4</td>
<td></td>
<td></td>
<td>+</td>
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<tr>
<td>Ceramide</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>8-hydroxyeicosapentaenoic acid</td>
<td></td>
<td>+</td>
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</tr>
<tr>
<td>Phytanic acid</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Hypolipidemic drugs</td>
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<tr>
<td>▪ Clofibric acid</td>
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<tr>
<td>▪ Ciprobific acid</td>
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<tr>
<td>▪ Gemfibrozil</td>
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<tr>
<td>▪ Wy-14,643</td>
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<td>+</td>
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<tr>
<td>▪ Eicosatetraynoic acid</td>
<td>+</td>
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<tr>
<td>▪ Benzalibric acid</td>
<td>+</td>
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<td>▪ GW501516</td>
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<tr>
<td>Indomethacin</td>
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<td>Ibuprofen</td>
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<tr>
<td>Troglitazine</td>
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<tr>
<td>Rosiglitizone</td>
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<tr>
<td>Pioglitazone</td>
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<td></td>
<td>+</td>
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<tr>
<td>Fatty acyl dehydrogenase inhibitors</td>
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<td></td>
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<tr>
<td>Carnitine palmitoyl transferase 1 inhibitors</td>
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<tr>
<td>Phthalates</td>
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<td>+</td>
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<tr>
<td>Mono-2-ethylhexylphthalate</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Dichloro and trichloroacetic acid</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Tributyltin</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Halogenated analogs of Bisphenol A</td>
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<td>+</td>
</tr>
</tbody>
</table>

* There are few antagonists of PPARs. Typical antagonists are pharmacological ligands such as SR-202, GW9662, JTP-426467, HL005 or biphenol-A-diglycidyl ether (BADGE) (Dworzanski et al., 2010; Li et al., 2009; Lu et al., 2011; Nakano et al., 2006; Nishiu et al., 2006; Rieusset et al., 2002). Tributyltin oxide has recently been shown to block PPAR\(\alpha\) and PPAR\(\beta/\delta\) activity in fish (Collier et al., 2011).
7.3 Consequences of Disruption

195. 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 have a BMI greater than 25, and therefore are considered overweight (Janesick and Blumberg, 2011). 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%), American Samoa (75%), Panama (35%), Mexico (24.2%), the United Kingdom (23%), the United Arab Emirates (34%), Nauru (79%), Kiribati (51%), Israel (23%), Greece (23%), and Chili (22%) (World Health Organization, 2011). Approximately 200,000,000 men and 300,000,000 women worldwide are obese (World Health Organization, 2011), and obesity rates are growing rapidly in parts of Europe, Latin America, the Caribbean, and Middle East (Berghöfer et al., 2008; Hatch et al., 2010).

196. 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 causes 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.

197. In 2002, a review paper suggested that weight gain may be caused by environmental toxicants. It suggested weight gain as a side effect of specific pharmaceuticals or toxicants was being overlooked by toxicologists primarily interested in weight loss as a symptom of toxicity (Baillie-Hamilton, 2002). A few years later, the term “obesogen” was coined (Grun et al., 2006). Another term used is “metabolic disruptor” (Casals-Casas et al., 2008; Casals-Casas and Desvergne, 2011). Both terms define a new subclass of endocrine disruptors that perturb metabolic signaling and energy (lipid) homeostasis, leading to increased weight, adipogenesis, and obesity. 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 potential consequences of PPAR disruption.

7.3.1 PPARα

198. 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 (Pyper et al., 2010). Additionally, PPARα attenuates inflammatory responses and, consequently, PPARα-null mice have prolonged inflammatory responses (Devchand et al., 1996).

199. In general, the list of PPARα’s functions appears positive. PPARα activation lowers triglyceride levels in the circulatory system, and reduces adiposity, which, in turn, improves insulin sensitivity (Chou et al., 2002; Guerre-Millo et al., 2000; Michalik et al., 2006). Because of this, PPARα activators such as the fibrate drugs reduce hypertriglyceridemia. PPARα-null mice are unable to respond to fibrate drugs and therefore, their hyperlipidemia does not improve (Michalik et al., 2006; Pyper et al., 2010). PPARα also protects against muscle and hepatic steatosis, including diet-induced steatohapatitis (Chou et al., 2002; Hashimoto et al., 2000; Ip et al., 2003). Furthermore, the anti-inflammatory effects of PPARα agonists have positive effects on the cardiovascular system (Berger et al., 2005).
200. However, PPARα ligands are peroxisome proliferators, and they promote liver carcinogenesis in rodent models (Holden and Tugwood, 1999; Palmer et al., 1998; Rao and Reddy, 1987). PPARα ligands have not been shown to cause mutations, and thus, are considered nongenotoxic carcinogens. Current hypothesis 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 (Cajaraville et al., 2003; Gulick et al., 1994). Further, peroxisome proliferators increase hydroxylation of fatty acids by inducing CYP 4A family members (Muerhoff et al., 1994). This, in turn, increases reactive oxygen species and perturbs eicosanoid homeostasis. These changes may play a role in cell proliferation and carcinogenesis.

201. Nevertheless, there is currently little evidence that PPARα ligands and peroxisome proliferators cause liver cancer in humans, and peroxisome proliferators are not considered human carcinogens (Gonzalez and Shah, 2007; Holsapple et al., 2006). 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 (Yu et al., 2001). 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 hypertrophy while still mediating many of the functions ascribed to PPARα (Qian et al., 2008). Taken together, peroxisome proliferators are not considered carcinogens in humans (Gonzalez and Shah, 2007; Holsapple et al., 2006).

202. PPARα is also expressed during fetal development (Abbott, 2009), and therefore there is the potential for developmental effects. For example, both PFOS and PFOA activate PPARα, and initiate development defects. PFOA reduced survival, delayed eye opening, and caused a decrease in body weight (Abbott, 2009); 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 and one that requires further study.

7.3.2 PPARβ/δ

203. PPARβ/δ controls energy homeostasis by regulating genes involved in fatty acid catabolism and adaptive thermogenesis in the heart, skeletal muscle, liver, and fat (Barish et al., 2006; Feige et al., 2006). 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 (Jawerbaum and Capobianco, 2011; Michalik et al., 2006; Michalik and Wahli, 2006; Tan et al., 2007). Lastly, PPARβ/δ has anti-inflammatory properties mediated on macrophages (Barish et al., 2006; Michalik et al., 2006).

204. For example, in animal models, PPARβ/δ agonists reduce weight gain caused by high-fat diet and, in turn, maintain insulin sensitivity, probably by increasing skeletal muscle fatty acid catabolism and thermogenesis (Wang et al., 2003). Whether PPARβ/δ ligands reduce weight gain in humans in not known (Barish et al., 2006). One of the most promising aspects of PPARβ/δ activation is the increase in HDL coupled with lower cholesterol and triglycerides (Oliver et al., 2001). Several therapeutics reduce cholesterol or triglycerides, but few therapeutics positively affect HDL levels (Barish et al., 2006). Furthermore, the gain of function VP16-PPARα/β 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 (Wang et al., 2003). Thus, PPARβ/δ has positive actions on triglycerides, cholesterol, HDL, and weight gain in rodent and rhesus monkey models.

205. 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 (Barish et al., 2006; Tanaka et al., 2003; Wang et al., 2004). 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 (Kelley et al., 1999). Concurrently, PPARβ/δ ligands increase fatty acid oxidation in the heart, which also primarily uses fatty acids for energy (Stanley et al., 2005). This increases heart contractile function (Cheng et al., 2004).

206. 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 whether there affects are adverse.

7.3.3 PPARγ

207. PPARγ is crucial in adipose tissue differentiation and adipocyte function, such as fat storage and energy dissipation (Desvergne and Wahli, 1999; Feige et al., 2006; Willson et al., 2001). PPARγ is pivotal in glucose metabolism because it improves insulin sensitivity (Semple et al., 2006). Therefore, the PPARγ ligands such as the thiazolidinediones improve insulin sensitivity and reduce hyperglycemia and are useful treatments for type II diabetes (Willson et al., 2001). Inflammation is also impeded by PPARγ agonists, thus providing additional roles for the zolidinediones in improving atherosclerosis and diabetes (Michalik et al., 2006).

208. Unlike the other PPAR receptors, PPARγ activity has some clear downsides. Heterozygous PPAR γ+/- mice show reduced weight gain after treatment with a high-fat diet (Willson et al., 2001). 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 (Ristow et al., 1998). PPARγ antagonists prevent weight gain in high-fat diet treated rodents (Nakano et al., 2006; Nishiu et al., 2006; Rieusset et al., 2002). In addition, a side effect of the prolonged use of thiazolidinediones is weight gain (Willson et al., 2001), but thiazolidinediones are continually used because the benefits of the glitazones outweigh their side effects for persons with type II diabetes.

209. 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 (Willson et al., 2001). 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 (Masud and Ye, 2003; Razquin et al.,
210. Chemically induced PPARγ activity causes obesity as determined by studies that demonstrate that glitazones (thiazolidinediones) increase weight gain (Larsen et al., 2003; Rubenstrunk et al., 2007; Willson et al., 2001). In addition, environmentally relevant PPAR agonists increase weight gain and lipid deposition, and a majority of these show activity towards PPARγ (Grun et al., 2006; Grun and Blumberg, 2009, 2009; Janesick and Blumberg, 2011; Li et al., 2011) with some showing additional PPARα activity (Abbott et al., 2007; Abbott, 2009; Grun and Blumberg, 2009; Maloney and Waxman, 1999). Furthermore, the urinary concentrations of phthalate metabolites are associated with increased waist circumference and insulin resistance (Stahlhut et al., 2007). The promiscuous nature of some of these chemicals such as the phthalates for multiple nuclear receptors complicates their assessment (Grun and Blumberg, 2009), 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 (Grun et al., 2006; Grun and Blumberg, 2009; Janesick and Blumberg, 2011; Li et al., 2011)

211. Interestingly, PPARγ activity and adipocytes differentiation can be activated without binding, and instead with increased PPARγ expression. The environmental estrogen, bisphenol A, increases PPARγ expression, and in turn alters IGF-1 expression and increases early adipogenesis in rats (Kwintkiewicz et al., 2010; Somm et al., 2009). Furthermore, it may be involved in promoting adipogenesis in 3T3-L1 cells (Sargis et al., 2010). Halogenated analogs of bisphenol A 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 adipocytes differentiation in 3T3-L1 cells, indicating that they are potential obesogens (Riu et al., 2011)

212. 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 (Ryu et al., 2007). PPARγ also is associated with the proliferation and the inhibition of proliferation of certain cancers (Kim et al., 2011; Lu et al., 2011; WangWang et al., 2011; Zaytseva et al., 2011). Whether it improves or exacerbates the outcome is currently debated and may depend on specific conditions, and perhaps, even the type of cancer (Michalik et al., 2006). Overall, these are other potential adverse effect of PPARγ.

### 7.3.3.1 PPAR Disruption in Wildlife

213. 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 (Cajaraville et al., 2003; Ciolek and Dauça, 1991). Exposure to a diverse set of chemicals, including PAHs, phthalates, alkylphenols and pesticides, has included acyl-CoA oxidase or peroxisome proliferation in fish (Cajaraville et al., 2003). For example, the organochlorine’s endosulfan and dieldrin and the organophosphate disolfoton caused peroxisome proliferation in rainbow trout and gilthead sea bream (Arnold et al., 1995; Pedradas et al., 1996). 2,4-D treatment increased peroxisome proliferation in mummichogs (Ackers et al., 2000).

214. Clofibrate increased peroxisome proliferation with *Rana esculenta* showing greater sensitivity than *X. laevis* (Ciolek and Dauça, 1991). In addition, Clofibrate and gemfibrozil induce embryonic malabsorption syndrome in zebrafish, resulting in small embryos (Raldúa et al.). This effect is reversible when the drugs are 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 processes (Prindiville et al., 2011).

215. 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 (Cangialosi et al., 2011). Taken together, PPARα activators have measurable effects on fish and amphibian species; however, the adverse outcomes of these exposures are poorly understood.

216. Few studies have addressed the effects of PPARγ agonists on fish or amphibians, but there are a few. TBT promotes adipogenesis in *Xenopus laevis* (Grun et al., 2006). 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*) (Cerio et al., 2011). 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 (Meador et al., 2011). Interestingly, tributyltin oxide (TBTO) inhibits PPARα and PPARβ/δ activity (Colliar et al., 2011). 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 (see Table 7-2). In summary, there have been few through studies of PPAR agonists on environmentally relevant species; therefore, the potential adverse effects on these species is 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.

7.4  **In vitro Screening Assays**

217. There are several methods for measuring the binding or activation of PPAR receptors. In addition, the 3T3-L1 adipocyte cell differentiation assay is a relatively brief method for measuring a functional endpoint (Green and Kehinde, 1975) often caused by PPAR activation, especially activation of PPARγ (Mukherjee et al., 2000). A brief overview of several *in vitro* screening methods is provided below.

7.4.1  **Ligand Binding Assays**

218. The ligand binding assay is the classical assay that demonstrates interaction between a chemical and a target receptor. However, it does not demonstrate a functional response. For example, it does not guarantee that the ligand activates or inactivates a specific receptor. On the other hand, it does demonstrate a physical interaction between receptor and ligand. It often uses radioactivity, but can be done with other markers such as biotin. Other examples of similar assays that demonstrate direct binding and affinity are the fluorescence resonance energy transfer (FRET) assay, the scintillation proximity assay, and surface plasmon resonance. Examples these assays can be found in the literature (Baldwin et al., 1998; Korach et al., 1988; Li et al., 2009; Lin et al., 1999; Moore et al., 2000; Nichols et al., 1998; Parks et al., 1999; Yang et al., 2007; Yue et al., 2005).

7.4.2  **Transactivation Assays**

219. The transactivation assay is the classical reporter assay that demonstrates functional activation of a nuclear receptor by a specific compound (Gulick et al., 1994). Sometimes it is assumed
that transactivation occurs because of ligand binding, which is often the case; however, it is not necessarily so. For example, PPARs can be activated by ligand-independent means through phosphorylation (Juge-Aubry et al., 1999; Zhang et al., 1996), and in the case of PPARγ inhibited by phosphorylation (Camp and Tafuri, 1997; Hu et al., 1996). Ligand-independent phosphorylation is not unique to PPARs and as other nuclear receptors such as the estrogen receptor (Kato et al., 1995) and the constitutive androstane receptor (Kawamoto et al., 1999; Kobayashi et al., 2003) also show ligand-independent activation through different signal transduction cascades.

220. 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 (Baldwin and Roling, 2009; Choi et al., 1997; Collier et al., 2011; Giguère et al., 1986; Gulick et al., 1994; Tzameli et al., 2000). 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.

221. Transactivation assays can be modified by the addition of cofactors and 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) (Krey et al., 1997).

222. 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) (Desvergne and Wahli, 1999). Interestingly, chemicals shown to activate PPARs are almost always ligands (Desvergne and Wahli, 1999).

### 7.4.3 3T3-L1 Cell Differentiation Assay

223. 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 (Green and Kehinde, 1975). 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.

### 7.5 In Vivo Screening and Testing Assays

#### 7.5.1 Peroxisome Proliferation

224. The key biomarker for PPARα activation is peroxisome proliferation (Holden and Tugwood, 1999; Palmer et al., 1998; Rao and Reddy, 1987), and therefore this liver phenotype can be used to demonstrate PPARα activation in vivo. 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
PPAR\(\alpha\) mice are also available, and these could be used to reduce the extrapolation from rodents to humans.

7.5.2 Lipid Accumulation

225. The key biomarker or physiological change induced by an obesogen is increased weight gain, especially increased weight gain through lipid accumulation. Considering the incredible increase in obesity over the past 30 years, this is a key biomarker for a number of chemicals not just PPAR\(\gamma\) 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* (Grun et al., 2006). We foresee techniques such as Dual-emission X-ray absorptiometry (DXA) (St-Onge et al., 2004) being helpful in the diagnosis of chemically induced obesity. Conditional knockouts and gain of function transgenics (Sugii et al., 2009) have been produced, and some of these may help provide further incite on the physiological effects of metabolic disruptors.

7.5.3 Microarrays

226. 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 providing key insight into the mechanism of action of numerous chemicals, including PPAR activators (see Table 7-3; Carlson et al., 2011; Cerio et al., 2011; Hamadeh et al., 2002; Omura et al., 20007; Pennie, 2000; Rosen et al., 2008).

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

7.6 Challenges and Limitations

227. Several specific challenges have been addressed throughout the review as they pertain to specific receptors or methods. However, there are two primary challenges facing PPAR/metabolic disruptors.

228. First, there are significant species differences in responses. For example, peroxisome proliferation has not been observed in humans because humans express PPAR\(\alpha\) at much lower levels than rodents (Qian et al., 2008). Thus, activation of PPARs in rodents does not necessarily reflect similar physiological perturbations in humans. Furthermore, fish PPAR\(\gamma\) 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.

229. Second, we have to decide how to interpret the data generated to decide whether specific pharmaceuticals, or environmental and industrial chemicals need further regulation. For example, are glitazones in the environment a potential hazard and at what concentrations? To answer these questions, prioritization is necessary. Some environmentally relevant chemicals have been shown to activate PPARγ. Are there others and what are they? What are the physiological effects that we should be most concerned about in the human population? Considering all of the causes of obesity is PPARγ activation a pivotal response? What are critical levels of PPAR agonists in the environment? Should regulations be based on the effects of mixtures considering the possibility of additive effects from multiple activators? Of course, significant screening is necessary to determine how widespread PPAR activation is by environmental chemicals and pharmaceuticals released into the environment. Furthermore, more basic research on the physiological functions of PPARs is needed, especially for PPARβ/δ.
8. Epigenetics and Endocrine Disruption

This chapter (Chapter 8 on Epigenetics and Endocrine Disruption) is being revised separately on the basis of the comments received at and after the EDTA AG meeting in April 2011. You are not requested to comment on chapter 8 as included in the draft DRP but you’ll receive a separate request for comments by the end of July 2011.

8.1 Introduction

230. The mechanism by which the group of chemicals referred to as ‘endocrine disruptors’ exert their phenotypic effects remains unknown, but there is emerging evidence that dysregulation of the cell’s epigenome is involved. This section reviews our current understanding of the intersection of these two fields of research and proposes avenues of exploration that will form the foundation for definitive testing of this relationship.

8.2 Definitions

8.2.1 The Epigenome

231. 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 two chromosomes identical 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.

232. Some of the molecular mechanisms implicated in allelic silencing included methylation of DNA (Sapienza 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 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 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.

233. “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.

234. Acknowledging that the use of the term epigenome or epigenomic is inherently flawed in terms of its origins, this section 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.

8.2.2 Epigenomic Regulatory Mechanisms

The molecular mechanisms believed to mediate epigenetic and transcriptional regulation are diverse (summarized in Table 8-1).

Table 8-1. Examples of Molecular Regulators of the Epigenome

<table>
<thead>
<tr>
<th>Molecular Mediator</th>
<th>Example</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone post-translational modifications</td>
<td>Histone H3 lysine 9 trimethylation (H3K9me3), a repressive mark</td>
<td>(Hiragami-Hamada et al., 2009)</td>
</tr>
<tr>
<td>Histone variants</td>
<td>Histone Macro H2A.1</td>
<td>(Bernstein et al., 2008)</td>
</tr>
<tr>
<td>Nucleosome positioning</td>
<td>Nucleosome-free regions at gene promoters</td>
<td>(Hartley and Madhani, 2009)</td>
</tr>
<tr>
<td>Chromatin looping</td>
<td>Kit regulation by Gata1/Gata2</td>
<td>(Jing et al., 2008)</td>
</tr>
<tr>
<td>DNA modifications</td>
<td>Cytosine methylation</td>
<td>(Klose and Bird, 2006)</td>
</tr>
<tr>
<td>DNA structural variation</td>
<td>R-loop formation</td>
<td>(Roy et al., 2008)</td>
</tr>
<tr>
<td>RNA-mediated</td>
<td>Antisense RNA transcription</td>
<td>(Beiter et al., 2009)</td>
</tr>
</tbody>
</table>

235. 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 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 is little evidence for such mechanisms in mammalian cells at present (Kim et al., 2006; Morris et al., 2004).

236. 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 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. This maintenance methyltransferase function allows a methylation mark in a parent cell to be maintained in both daughter cells.

237. 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 et al., 2009). RNA-mediated effects such as paramutation, best described in plants (Chandler, 2007), have been observed in mice (Rassoulzadegan 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 et al., 2006).

238. 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.

8.2.3 Influences Exerted by Epigenomic Regulatory Mechanisms

239. 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.

240. 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 a repressive mark, associated with heterochromatin on a cytological scale (Peters et al., 2002) and with gene silencing when present at a promoter. However, the same modification is found to be enriched in the bodies of actively-transcribed genes (Vakoc et al., 2005), the opposite correlation. 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 et al., 2009). This contextual information is important when defining relationships of epigenomic regulatory marks and transcription.

241. 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 et al., 2009), and has been more precisely mapped to areas of open chromatin in yeast (Kauppi 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 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 et al., 2010), indicating that even cytological-scale morphology has regulatory input by these epigenomic mediators.

242. Recently there has been an unexpected relationship revealed between chromatin organization (Vakoc et al., 2005) or DNA methylation (Laurent 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 et al., 2009) and DNA methylation observed at intron/exon boundaries have been shown to be distinctive (Laurent 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.
243. 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 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.

244. 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. In Table 8-2 we list these initiatives and web-based resources for the reader to explore further.

### Table 8-2. Large-scale Studies Studying Epigenomic Organization

<table>
<thead>
<tr>
<th>Project</th>
<th>Abbreviation</th>
<th>Web Resources</th>
</tr>
</thead>
</table>
| ENCYclopedia Of DNA Elements                   | ENCODE, modENCODE | • [http://www.genome.gov/10005107](http://www.genome.gov/10005107)  
• [http://genome.ucsc.edu/ENCODE/](http://genome.ucsc.edu/ENCODE/)  
• [http://www.modencode.org/](http://www.modencode.org/)  
• [http://www.epigenomebrowser.org/](http://www.epigenomebrowser.org/)  

245. These projects are now productive and provide insights into how epigenomes are organized, and how epigenomic information interacts with genetic polymorphism (Kasowski et al., 2010; McDaniel 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.

### 8.2.5 Genome-Wide Assays: The Transition from Microarrays to Massively-Parallel Sequencing

246. When performing a genome-wide study, in essence they involve 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 the relative amount of sequence mapped to a specific gene or location.

247. 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, we have 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 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.

248. 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 9.

8.2.6 The Problem of Choice in Epigenomic Assays

249. When cells with an identical genome are compared for epigenomic differences following exposure to different toxins, it is apparent even from Table 8-1 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 8-1 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 on histone variants are considered, the positioning of the nucleosomes they assemble, the influence DNA methylation of the DNA they package, and so on. 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.
250. In practice, the choice is often constrained to a focus on DNA methylation studies for a number of reasons. There is a more general familiarity with the assays involved, the sample requirements are generally less onerous (in terms of quantity and preparation) than for RNA or chromatin-focused studies, and the assays are demonstrably quantitative, something that has yet to be shown for ChIP followed by MPS (ChIP-seq).

251. A significant problem with DNA methylation is that we don’t really know how to interpret many of the non-promoter changes we observe, 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 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.

8.3 Potential Effects.

252. The obvious pathogenetic mechanism for endocrine disruptors is through their action on their cognate sex steroid receptors. This is enough to account for many of the effects of these agents on animal phenotypes, including male fertility (Guerrero-Bosagna and Skinner, 2009), anatomical structures and histological organization of male reproductive organs (Svechnikov et al., 2010), ovarian development (reviewed in (Uzumcu and Zachow, 2007)) and female reproductive tract cancer (Ma, 2009). This accounts for much of the recognized phenotypic spectrum of endocrine disruptors, prompting the question whether the epigenome is worth studying at all?

253. 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 3) that offer more direct evidence for mechanistic associations.

254. 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.
Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors

256. 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.

### 8.4 Evidence For Endocrine Disruption.

257. 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 8-3 to be presented as a summary of the state of this field.

**Table 8-3. Genes Regulated by DNA Methylation (from (Zhang and Ho, 2011))**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450scc</td>
<td>CYP11A1</td>
</tr>
<tr>
<td>3β-hydroxysteroid dehydrogenase</td>
<td>HSD3B1/2</td>
</tr>
<tr>
<td>17α-hydroxylase</td>
<td>CYP17A1</td>
</tr>
<tr>
<td>17β-hydroxylase</td>
<td>HSD17B3</td>
</tr>
<tr>
<td>Vitamin D synthesis</td>
<td>CYP27A1/B1</td>
</tr>
<tr>
<td>Androgen receptor</td>
<td>AR</td>
</tr>
<tr>
<td>Oestrogen receptor 1</td>
<td>ESR1</td>
</tr>
<tr>
<td>Oestrogen receptor 2</td>
<td>ESR2</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>PGR</td>
</tr>
<tr>
<td>Glucocorticoid receptor</td>
<td>NR3C1</td>
</tr>
<tr>
<td>Mineralocorticoid receptor</td>
<td>NR3C2</td>
</tr>
<tr>
<td>Retinoic acid receptor α</td>
<td>RARA</td>
</tr>
<tr>
<td>Retinoic acid receptor β</td>
<td>RARB</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>SST</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>VAP</td>
</tr>
<tr>
<td>Melanocyte-stimulating hormone</td>
<td>POMC</td>
</tr>
<tr>
<td>Secretin</td>
<td>SCT</td>
</tr>
<tr>
<td>Insulin</td>
<td>INS</td>
</tr>
<tr>
<td>Leptin</td>
<td>LEP/OB</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>OXTR</td>
</tr>
<tr>
<td>Follicle stimulating hormone</td>
<td>FSHR</td>
</tr>
<tr>
<td>Thyroid stimulating hormone</td>
<td>TSHR</td>
</tr>
<tr>
<td>Insulin-like growth factor 2</td>
<td>IGF1R/IGF2R</td>
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</tbody>
</table>

258. The 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 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. 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 thyroid hormone 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 RXRs it activates transcription by recruiting the histone acetyltransferases PCAF and CBP (Zhang and Lazar, 2000). This example represents a much broader picture, with almost 300 nuclear receptor co-regulators now documented (O’Malley et al., 2008) and multiple examples of ligand-dependent effects to activate or repress transcription (Kato et al., 2011). Genes that represent specific targets of oestrogen receptor alpha have been identified using the chromatin immunoprecipitation approach (Jin et al., 2004; Lin et al., 2007), allowing insight into the downstream effectors of hormonal signaling.

259. 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 our group studied a rat model of IUGR and quantified cytosine methylation throughout the genome in beta islet cells from the pancreas of young adult rats, we 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 (ThompsonFazzari 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.

260. There are examples of endogenous hormones and endocrine disruptors having effects mediated by different epigenetic and transcriptional regulatory processes. The endocrine disruptor bisphenol A has been a major focus of investigation for some time, given its broad exposure within the population (Calafat et al., 2008) and the observed effects in animal models on the development of breast and prostate (reviewed in (Weng et al., 2010)). Interestingly, there is little published to demonstrate epigenome-wide effects of bisphenol A, with several reports focusing on individual loci (Bromer et al., 2010; Dolinoy et al., 2007; Weng 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 et al., 2008), the other testing prostate tissue using Methylation-Sensitive Restriction Fingerprinting (MSRF) (Ho 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.

261. 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) 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 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 11.3) was described the primary data were not presented in that original study (Anway et al., 2005). 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 et al., 2010). No locus-specific studies were performed in this project.

262. 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 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 et al., 2009), no comparable experiments have been described for endocrine disruptors.

8.5 Assay Methods

8.5.1 The Problem of Choice in Epigenomic Assays.

263. 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.

264. As the comprehensiveness and quantitative capabilities of genome-wide assays improved while costs decreased, the focus has shifted towards what can be described as genome-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.

265. 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.

266. First of all, epigenetic dysregulation events are believed to be somatic rather than constitutional, 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).

267. 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.

268. We have also 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 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% (ThompsonFazzari et al., 2010), and a recent paper testing liver epigenomes of mice whose fathers were fed different diets a comparable value (Carone 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.

269. 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. We have determined that the use of the MSCC (Ball et al., 2009) or our HELP-tagging (Suzuki 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 et al., 2010; Kasowski et al., 2010; McDaniell et al., 2010). It is also notable that cytosine methylation appears to be influenced by age (Fraga et al., 2005; ThompsonAtzmon et al., 2010) and sex (Sarter et al., 2005), combining to require that cohorts should be matched in terms of self-reported ethnicity, age and sex in order to reduce these potential sources of variability.
270. 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 et al., 2007), and that these loci may be preferentially involved in mediating disease states, as we found in our 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 et al., 2008; Yamashita 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.

271. 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.

8.5.2 Designing an Epigenome-Wide Association Study Of Endocrine Disruptors.

272. The discussions of section 5 give us some guidelines about how we might go about searching for the effects of endocrine disruptors on the epigenome. As a first step we would 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 toxin exposure (section 7).

273. 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.

274. 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.
275. 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 et al., 2011), testing transposable elements like long or short interspersed nuclear elements (LINEs, SINEs) with bisulphite sequencing (Yang et al., 2004), or performing luminometric methylation analysis (LUMA) (Karimi et al., 2006), to name a few. If a more functional test is required, the viable yellow (Avy) mouse model has characteristics that have caused it to be described as an ‘epigenetic biosensor’ (Dolinoy, 2008). 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 disruptors (Dolinoy 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).

276. Cell culture systems represent the mainstay of many of the current studies of endocrine disruptors effects upon the epigenome. A problem with cultured (Meissner et al., 2008) and transformed (Wild 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, but potentially useful if the culture conditions are kept identical between conditions being compared.

8.5.3 Is The Use Of Model Organisms Necessary?

277. 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 et al., 2007; Meissner et al., 2008). There are thus numerous advantages to the use of animal models.

278. One especially advantageous reason for using animal models is the ability to pursue the transgenerational effects of endocrine disruptors (Anway et al., 2005; Anway and Skinner, 2006; Crews et al., 2007; Guerrero-Bosagna and Skinner, 2009). This has already proven interesting in terms of studies of epigenetic organization in testes (Anway 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 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 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 et al., 2001).

279. So while rodent models have limitations in how they represent human exposures (Stokes, 2004), there are many factors that suggest that they represent a practical, short-term means of gaining insights into whether and how endocrine disruptors influence epigenomic organization in vivo.
8.5.4 What are The Potential Future Advances Facilitating New Approaches?

280. There is reason for optimism regarding our ability to use technology more effectively to gain insights to the epigenomic 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, probably 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 sequencing costs per basepair are dropping at a substantially faster rate. 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 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.

281. 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 et al., 2005) and paradoxically increased cytosine methylation (Ball 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.

8.6 Challenges

282. 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. 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.

283. The analysis challenge is also substantial. 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.

284. 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 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.

8.7 Conclusions and Testing Recommendations

8.7.1 Conclusions

285. 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 relative 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.

286. 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.

Table 8-4. OECD Tests that Could Potentially Be Adapted for Epigenomic Studies of Effects of Endocrine Disruptors

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Test</th>
<th>Description</th>
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<tbody>
<tr>
<td>General exposure studies</td>
<td>Test 451, 452, 453</td>
<td>Carcinogenicity Studies, Chronic Toxicity Studies, Combined Chronic Toxicity/Carcinogenicity Studies</td>
</tr>
<tr>
<td>Post-mitotic cell studies</td>
<td>Test 424</td>
<td>Neurotoxicity Study in Rodents</td>
</tr>
<tr>
<td>Pre-natal effects</td>
<td>Test 414</td>
<td>Prenatal Development Toxicity Study</td>
</tr>
<tr>
<td>Transgenerational effects</td>
<td>Tests 415, 416, 421, 422</td>
<td>One- and Two-Generation Reproduction Toxicity, Reproduction/Developmental Toxicity Screening Test, Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test</td>
</tr>
<tr>
<td>In vitro systems</td>
<td>Test 473</td>
<td>In vitro Mammalian Chromosome Aberration Test</td>
</tr>
</tbody>
</table>
8.7.2 Testing Recommendations

287. We therefore have some recommendations for testing, based on existing test systems used by the OECD. We list them 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.

8.7.2.1 General Exposure Studies

288. There are as many as three tests that appear suitable for testing epigenetic effects of exposures throughout the body. Tests 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.

8.7.2.2 Post-Mitotic Cell Studies

289. Test 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.

8.7.2.3 Pre-Natal Effects

290. Test 414 (Prenatal Development Toxicity Study) involves the exposure to animals of agents during pregnancy, testing the foetus at term for abnormalities, while Test 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.

8.7.2.4 Transgenerational Effects

291. A genuinely transgenerational study requires looking as far as the F3 generation (Skinner, 2008). Current test systems only proceeds to the F2 generation, so available test systems are not going to be definitive in testing for transgenerational effects, and will at best generate indicative, preliminary insights. There exist four tests that may allow such preliminary testing for transgenerational effects mediated by epigenetic dysregulation. Tests 415 and 416 (One- and Two-Generation Reproduction Toxicity), test 421 (Reproduction/Developmental Toxicity Screening Test) and test 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 two-generation test (416) could allow multiple tissues to be sampled in offspring of parents exposed to the agent of interest, allowing screening for inherited epimutations. The introduction of the new Extended One-Generation Reproductive Toxicity Study is likely to provide yet another test that can be used to look for transgenerational effects. Following up on the studies described in section 4 that identified transgenerational effects of endocrine disruptors, sperm cells would be candidates for analysis, but the timing of exposure of the gravid mother or pups is critical in terms of risk of the infertility phenotype (Anway et al., 2005) and should be taken into account in these studies.

8.7.2.5 In-vitro Systems

292. A modification of test 473 (In vitro Mammalian Chromosome Aberration Test) that left out the use of metaphase-arresting substances in exposed cells could be used to screen for epigenetic effects.
8.7.3 Potential New Test Systems

293. 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 may add utility. The viable yellow (A\textsuperscript{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 useful mouse resource, however. The Axin 1 fused (Axin1\textsuperscript{Fu}) mice also have a visible phenotype that is responsive to influences perturbing the epigenome (Waterland 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 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.

294. The other possible 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 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 exciting 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.

8.7.4 Validation of Tests

295. 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.

296. 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 et al., 2005) or Qiagen’s Pyrosequencer (Fakhrai-Rad et al., 2002) can perform this measurement highly quantitatively.

297. or 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.

298. 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.
299. 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) or diet (Niculescu and Zeisel, 2002). Functional validation remains an elusive component of current studies of epigenomic dysregulation.
9. Summary, Conclusions, and Recommendations

9.1 Summary and Conclusions

300. 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. In this DRP, we provide a discussion of those pathways that have undergone evaluation for susceptibility to endocrine disruptors and describe assays used to assess potential disruption of these pathways.

301. 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 (Shulman and Mangelsdorf, 2005). Age-adjusted estimates indicate that approximately 34% of the US population, over 19 years of age, meet the criteria for metabolic syndrome (Ervin, 2009). Metabolic syndrome has been associated with exposure to environmental chemicals, although the mechanistic relationship between exposure and disease outcome remains uncertain (Lee et al., 2007). The possibility must be considered that simultaneous disruption of multiple endocrine signaling pathways contribute to this condition.

302. 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 known association with metabolic syndrome (vom Saal and Myers, 2008).

9.1.1 Cross Talk Among Signaling Pathways

303. 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 (Section 7), 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 aryl hydrocarbon receptor (AhR) and are accordingly susceptible to the disrupting effects of AhR ligands, such as some dioxins and PCBs. Cross talk among signaling pathways add 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

304. Assays used to evaluate endocrine disruption described in this DRP fall within five major categories: binding assays, reporter gene assays, culture cell responses, microarrays, and in vivo apical endpoints. 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 6). 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, and these assays (Table 9-1) 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.

305. 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 and are time and cost effective.

306. 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 the assays often suffer from lack of reproducibility. Microarrays hold promise for the screening of chemicals for endocrine-disrupting properties; however, the approach may not be sufficiently developed for routine, validated use.

### Table 9-1. Some Commercially Available Reporter Gene Assay Kits for Use to Screen Chemicals for Interactions with Nuclear Receptors

<table>
<thead>
<tr>
<th>Nuclear Receptor</th>
<th>Commercial Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid (GR)</td>
<td>Panomics/Affymetrix</td>
</tr>
<tr>
<td></td>
<td>Indigo Biosciences</td>
</tr>
<tr>
<td></td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Androgen (AR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Estrogen (ER)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Progesterone (PR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Retinoic acid (RAR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Retinoid X (RXR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Thyroid (TR)</td>
<td>Indigo Biosciences</td>
</tr>
<tr>
<td>Vitamin D (VDR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Peroxisome Proliferator-Activated (PPAR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td>Aryl Hydrocarbon (AhR)</td>
<td>Qiagen/SABiosciences</td>
</tr>
<tr>
<td></td>
<td>Indigo Biosciences</td>
</tr>
</tbody>
</table>
Table 9-2. Cell-Based Assays Used to Assess Disruption of Endocrine Signaling Processes by Exogenous Chemicals

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Cells</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid (GR)</td>
<td>Corticotropes (primary)</td>
<td>ACTH release</td>
</tr>
<tr>
<td></td>
<td>Adrenal cortical (primary)</td>
<td>Adrenal hormone release</td>
</tr>
<tr>
<td>Somatotropic</td>
<td>Trout testicular (primary)</td>
<td>³H-thymidine incorporation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGF-1 binding</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Purkinje (primary)</td>
<td>Dendritic arborization</td>
</tr>
<tr>
<td></td>
<td>Granule cells (primary)</td>
<td>Neurite extension</td>
</tr>
<tr>
<td></td>
<td>GH3 (established)</td>
<td>Proliferation</td>
</tr>
<tr>
<td></td>
<td>FRTL-5 (established)</td>
<td>Iodine uptake</td>
</tr>
<tr>
<td>RXR/PPAR</td>
<td>3T3-L1 (established)</td>
<td>Differentiation</td>
</tr>
<tr>
<td></td>
<td>C3H10T1/2 (established)</td>
<td>differentiation</td>
</tr>
</tbody>
</table>

307. Many apical endpoints that have been described in this DRP could be added to currently recommend 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 be 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 9-3. Some Apical Endpoints that Could Be Applied to Currently Recommended Whole Organism Assays to Assess Disruption of Additional Endocrine Signaling Pathways

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>Stress responses</td>
</tr>
<tr>
<td>Androgen/estrogen</td>
<td>Reproductive behavior</td>
</tr>
<tr>
<td></td>
<td>GnRH and aromatase expression</td>
</tr>
<tr>
<td>Somatotropic</td>
<td>Plasma IGF-1 levels</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>Retinoid levels</td>
</tr>
<tr>
<td></td>
<td>Lipid levels</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Thyroid hormone levels</td>
</tr>
<tr>
<td></td>
<td>Thyroid gland histology</td>
</tr>
<tr>
<td></td>
<td>Thyroid hormone-responsive gene expression</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Plasma vitamin D and metabolites levels</td>
</tr>
<tr>
<td></td>
<td>Bone morphology</td>
</tr>
<tr>
<td>Fatty acid (PPAR)</td>
<td>Hepatic peroxisome proliferation</td>
</tr>
<tr>
<td></td>
<td>Lipid accumulation</td>
</tr>
</tbody>
</table>
9.1.3 Epigenetics

308. The supposition that early (e.g., in utero) exposure to an endocrine-disrupting chemical can result in dysfunction later in life (Thompson and Einstein, 2010) or even in subsequent generations (Anway et al., 2005) would require that methods be available to identify chemicals that are capable of inducing epigenetic modifications to an organism’s genome. Current evidence suggests a possible relationship between exposure to endocrine-disrupting chemicals and adverse modifications to the epigenome. Several OECD-recommended whole organism assays could be used as the foundation to evaluate epigenetic modifications to the genome. However, analytic approaches to assessing such changes remain limited. [To be further developed on the basis of the updated draft chapter on epigenetic mechanisms.]

9.2 Prioritization

309. All of the endocrine signaling pathways described in this DRP warrant consideration for inclusion in a chemical testing battery. The recommended priority for incorporation into a standard testing battery and the rationale for this recommended prioritization are provided below.

9.2.1 Highest Priority

310. Highest priority is given to the inclusion of the PPAR signaling pathway in a screening and testing battery. This pathway, typically activated by fatty acids, is clearly involved in lipid and glucose homeostasis, inflammation, and aspects of development. Perturbations in this pathway by environmental chemicals could have detrimental effects consistent with metabolic syndrome and other 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. 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.

9.2.2 Second Priority

311. We recommend that the next priority be the retinoid signaling pathway, particularly the RXR signaling pathway. 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 this screening battery. 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 animals’ exposure since AhR ligands can deplete retinoid levels and disrupt normal retinoid signaling.

9.2.3 Third Priority

312. Priority should be given to the thyroid signaling pathway with regards to screening assays that could address various aspects of this pathway. 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.

### 9.2.4 Fourth Priority

313. We recommend that the next priority be the incorporation of glucocorticoid signaling in a battery to assess endocrine disruption of chemicals. This pathway contributes to many physiological processes, including maintenance 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. Assays to consider in evaluating disruption of glucocorticoid signaling include reporter assays and culture cell responses. However, little precedent exists for the use of these assays in evaluating chemical effects on this pathway. Assay refinement and validation is required before these assays could be adopted for use in a screening battery.

### 9.2.5 Fifth Priority

314. The somatotropic axis holds promise in assessing endocrine disruption associated with chemical exposure because several endocrine signaling pathways converge on this pathway. According disruption of androgen, estrogen, corticosteroid, and thyroid signaling could be detected by alterations in circulating IGF-1 levels. 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 would require assay development and validation prior to use in a screening or testing battery since IGF-1 levels can be influenced by a various of exogenous factors (e.g., food, temperature, photoperiod) and the endpoint has not be extensively used to assess endocrine disruption.

### 9.2.6 Sixth Priority

315. Vitamin D plays important roles in the development and maintenance of various systems including bone, immune, cardiac, and neurological. Despite it 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 vitamin D receptor 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 is 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.

The prioritization of signaling pathway and recommended assays are summarized in Table 9-4.

### Table 9-4. Recommended Prioritization of Pathways and Assays to Be Incorporated Into the Screening and Testing Battery for the Detection of Endocrine-Disrupting Chemicals

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Priority</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR</td>
<td>First</td>
<td>Transactivation reporter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipocyte differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peroxisome proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lipid accumulation</td>
</tr>
<tr>
<td>Hormone</td>
<td>Stage</td>
<td>Assays/Endpoints</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>Retinoid</td>
<td>Second</td>
<td>RXR, RAR, AhR reporter assays, Adipocyte differentiation, Lipid accumulation, Serum retinoid levels</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Third</td>
<td>Transactivation reporter, Cell proliferation, Thyroid peroxidase inhibition, Iodide uptake</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Fourth</td>
<td>Transactivation reporter, ACTH release, Adrenal steroid, Synthesis, stress response</td>
</tr>
<tr>
<td>Somatotropic</td>
<td>Fifth</td>
<td>IGF-1 levels</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Sixth</td>
<td>Assay development required</td>
</tr>
</tbody>
</table>
10. Acknowledgements
11. References


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Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors


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